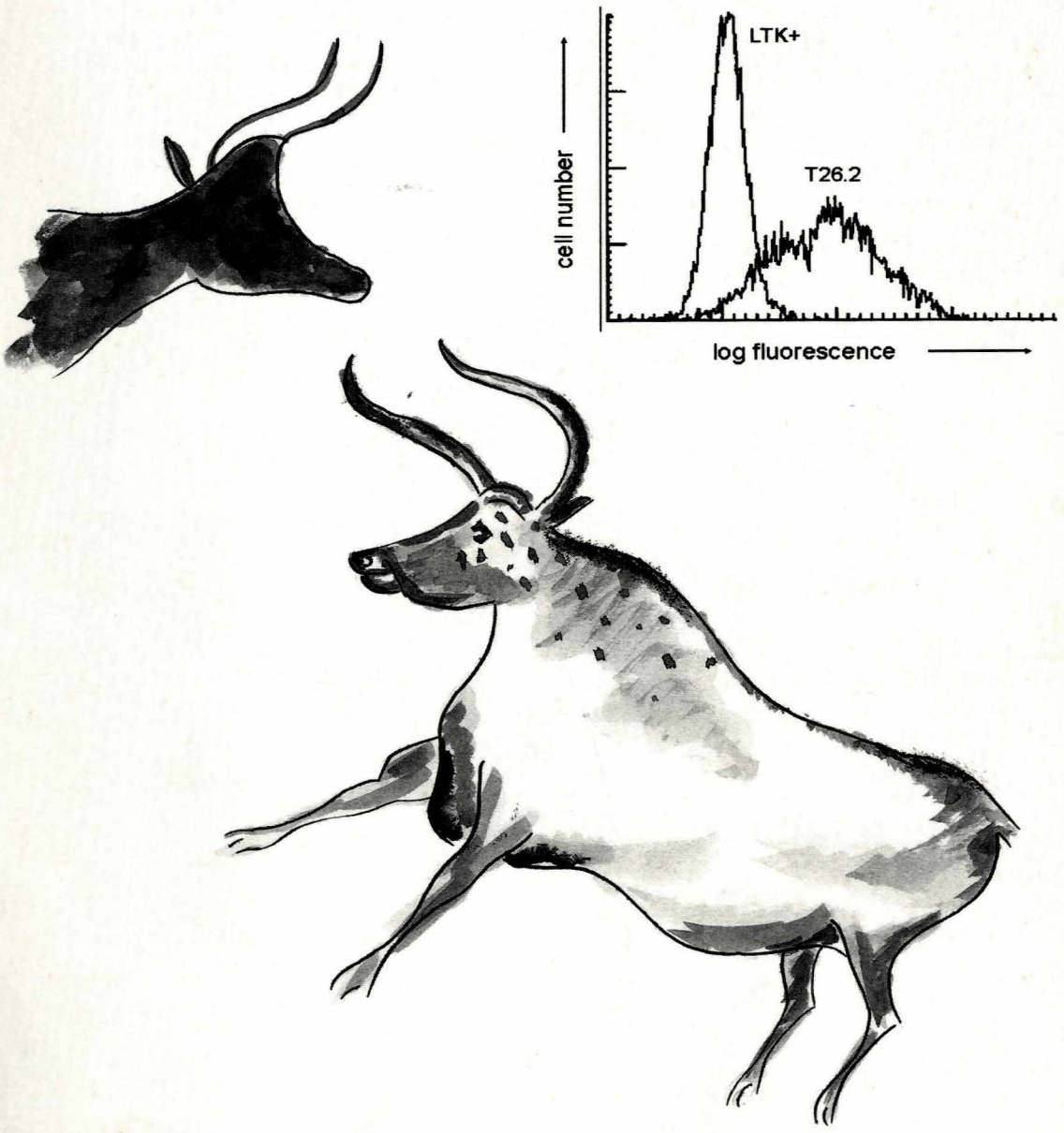
ANALYSIS OF THE IMMUNE RESPONSE OF RUMINANTS TO *CQWDRIA RUMINANTIUM* INFECTION

DEVELOPMENT OF AN INACTIVATED VACCINE



DOMINIQUE MARTINEZ

ANALYSIS OF THE IMMUNE RESPONSE OF RUMINANTS TO *COWDRIA RUMINANTIUM* INFECTION

DEVELOPMENT OF AN INACTIVATED VACCINE

ANALYSE VAN DE IMMUUNRESPONS VAN HERKAUWERS NA INFECTIE MET *COWDRIA RUMINANTIUM* ONTWIKKELING VAN EEN GEÏNACTIVEERD VACCIN

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus Prof.dr. J.A. van Ginkel, ingevolge het besluit van het College van Decanen in het openbaar te verdedigen op woensdag 21 mei 1997 des middags te 2.30 uur

door

Dominique Martinez
geboren op 31 maart 1955, te Rabat

Promoter: Prof.dr. B.A.M. van der Zeijst
Department of Bacteriology,
Institute of Infectious Diseases and Immunology,
Faculty of Veterinary Medicine,
Universiteit Utrecht.

Co-promoter: Dr. F. Jongejan
Department of Parasitology and Tropical Veterinary Medicine,
Institute of Infectious Diseases and Immunology,
Faculty of Veterinary Medicine,
Universiteit Utrecht.

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Martinez, Dominique

Analysis of the immune response of ruminants to *Cowdria ruminantium* infection. Development of an inactivated vaccine/ Dominique Martinez. - Utrecht: Universiteit Utrecht, Faculteit der Diergeneeskunde Thesis Universiteit Utrecht. - With ref. - With summary in Dutch and French.
ISBN 90-393-1050-5
Subject headings: *Cowdria ruminantium* / vaccination / heartwater / immunity

à Chantal
Guillaume
Benjamin & Margaux

CONTENTS

CHAPTER 1

INTRODUCTION

- Heartwater : an overview of the disease

- Vaccination and immunity against *Cowdria ruminantium* infection

- Aim and outline of the thesis

HEARTWATER : AN OVERVIEW OF THE DISEASE

Cowdriosis or heartwater is a tick-borne disease, affecting wild and domestic ruminants, which is endemic in SubSaharan Africa (Provost and Bezuidenhout, 1987) and several islands of the Indian Ocean (Uilenberg et al., 1983; Du Plessis et al., 1989; Flach et al., 1990) and the Lesser Antilles (Perreau et al., 1980; Birnie et al., 1984; Uilenberg et al., 1984) from where it threatens the American mainland (Barré et al., 1987). The causative organism is an obligate intracellular Gram-negative bacterium belonging to the order Rickettsiales which preferentially infects endothelial cells (Cowdry, 1925a) and can be found in neutrophils during the febrile phase of the disease (Logan et al., 1987).

Cowdriosis has been known for a long time, since the first record of a disease resembling heartwater was made by Louis Trichard in South Africa in 1838, who mentioned a fatal disease of sheep with central nervous signs following a massive infestation with ticks three weeks earlier (Neitz et al., 1947). Despite this very early description of the disease, the causative organism *Cowdria ruminantium* was described for the first time in the host and in the tick vector in 1925 by Cowdry (1925a,b), who classified it in the genus *Rickettsia*. Then, for a long period, research on heartwater has been neglected because of the failure to cultivate the pathogen *in vitro* or to adapt it to laboratory animals.

A revival in the research on heartwater followed the first description of the cultivation of *C. ruminantium in vitro* by Bezuidenhout et al., (1985). Since then, the methods of cultivation have been greatly simplified. Several lines of bovine, sheep, goat, and human endothelial cells from different vessels have been used successfully for the culture of many stocks of the parasite (Yunker et al., 1988; Byrom and Yunker, 1990; Martinez et al., 1990; 1993b; Jongejan et al., 1991d; Brett et al., 1992; Totté et al., 1993). Analysis of nutritional requirements has also been undertaken (Neitz and Yunker, 1996) to improve the cultivation of *C. ruminantium* which nevertheless remains a difficult task.

Studies on the ultrastructure of *Cowdria* in the host, the vector and *in vitro* by electron microscopy showed that the organisms live and multiply only in membrane-bound vacuoles within the host cell cytoplasm (Pienaar, 1970; Prozesky et al., 1986; Prozesky, 1987a; Kocan and Bezuidenhout, 1987). Jongejan et al. (1991d) described the life-cycle of *Cowdria* in endothelial cells as a *Chlamydia*-like development cycle. The cycle starts with the elementary body which represents the infectious form of the parasite. It adheres to and is engulfed by the endothelial cells where it remains within intracytoplasmic vacuoles, dividing by binary fission to produce a large colony named morula. Within the same vacuole, the organisms generally have similar size and shape. After 5 to 6 days, the disruption of the host cell leads to the release of

numerous elementary bodies that initiate a new infectious cycle (Jongejan et al., 1991d).

The exact taxonomic position of *Cowdria* has long been controversal. Based on the observations that a monoclonal antibody directed to the major outer membrane protein of *Chlamydia trachomatis* recognized *Cowdria* (Jongejan et al. 1991a), on the Chlamydia-like cycle of *Cowdria*, and on studies of numerical taxonomy conducted by Scott (1987), *Cowdria* was first considered to be close to the genus *Chlamydia*. Serological cross-reactions discovered with several *Ehrlichia* species (Du Plessis et al., 1987; Holland et al., 1987), conducted later to question this taxonomic position. Recently, analysis of 16S rDNA sequences have shown that *Cowdria* forms a tight phylogenic cluster with the canine pathogens *E. canis* and *E. ewingii*, the human pathogen *E. chaffeensis*, and the murine pathogen *E. muris* (Van Vliet et al., 1992; Dame et al., 1992; Allsopp et al., 1996). These results are however surprising regarding the known differences in host preferences and target cells.

So far, only ticks of the genus *Amblyomma* are known to be capable of transmitting *C. ruminantium* (Walker and Olwage, 1987). Although a possible role of mechanical transmission by insects cannot be excluded (Bezuidenhout, 1987), the distribution of heartwater corresponds with the distribution of *Amblyomma* ticks. In Africa, 5 species are involved in field transmission of the pathogen among which *A. variegatum* and *A. hebraeum* are indoubtedly the most important. Five other african species (*A. tholloni, A. sparsum, A. gemma, A. cohaerens, A. marmoreum*) have not been implicated in field outbreaks either because they are confined to forest areas or because of their host preference. Three American species (*A. maculatum, A. cajennense,* and *A. dissimile*) have proved to be experimental vectors of *Cowdria* (Uilenberg, 1982; Jongejan, 1992a, 1992b) and must be considered as a possible vectors if heartwater is introduced onto the American continent. Asian *Amblyomma* species have not yet been tested.

The transmission of *Cowdria* by ticks occurs after 1 to 3 days of feeding for nymphs and after 2 to 4 days for adult ticks (Bezuidenhout, 1988; Camus and Barré, 1992). Although transovarial transmission is possible (Bezuidenhout and Jacobsz, 1986), it plays a limited role compared with transstadial transmission. Intrastadial transmission by males which detach from an infected dead host and attach to another host has been achieved with *A. variegatum* and *A. hebraeum* (Andrew and Norval, 1989; Kocan et al., 1993) but probably plays a small role in the epidemiology of heartwater. Ticks are usually infected while feeding on a reacting or a carrier animal and infect another host after moulting to the following stage. In the tick, colonies first appear in the gut cells, and then in haemolymph and salivary glands from where the parasite is transferred into the vertebrate host during feeding (Kocan and Bezuidenhout, 1987). Tick infection rates in the field varies from as low as 1-2% (Camus, 1987) up to 20-36% (Norval et al., 1990). These variations may be partly

due to the method used to determine the tick infection rates but also to differences in susceptibility of species and populations of ticks to the infection by different stocks of *Cowdria*.

Following the infectious tick bite, clinical signs and mortality in the vertebrate hosts depend on their innate resistance and acquired immunity. The low susceptibility of local breeds in endemic areas and the high susceptibility of exotic breeds has been noted early (Neitz, 1939) and extensively reviewed in Camus et al. (1996). Today, cowdriosis is considered as the economically most important tick-borne disease of ruminants together with babesiosis, anaplasmosis, theileriosis and trypanosomiasis (Uilenberg, 1983; 1995). Susceptibility of taurine and zebu breeds appears to be equivalent, provided they originate from a non endemic region (Uilenberg and Camus, 1993). Considerable losses have been observed when these genotypes are imported for upgrading local breeds. However, even African zebu populations from *Amblyomma*-free regions can pay a heavy toll when moved in *Cowdria*-infected zones (Gueye et al., 1982; Logan et al., 1988). In endemic areas, local breeds benefit from natural selection over centuries and also from endemic stability. In these conditions, economic losses are considered rather low in cattle, although heartwater still remains a problem in small ruminants.

The differences in susceptibility of animals to heartwater implies that different forms of the disease varying from peracute to chronic can develop. In controlled tick-transmitted infections, the average incubation period varies from 1 to 5 weeks with an average of 2 weeks. After intravenous inoculation of infected blood, the average incubation period is 9-10 days in small ruminants and 12-13 days in cattle (Alexander, 1931; Uilenberg, 1983). When highly infected cultures are used, the incubation may be reduced to 3-4 days (Bezuidenhout et al., 1985). In fact, it appears that the incubation period depends on the dose of infective material, the stock of *C. ruminantium*, the route of inoculation and the ruminant species itself. In general goats are the most susceptible of the domestic ruminants followed by sheep and cattle.

As reviewed by Van de Pypekamp and Prozesky (1987), the course of the disease may range from peracute to mild depending on age, immune status, breed or the individual susceptibility and virulence of the *Cowdria* isolate. In susceptible animals, heartwater is usually an acute disease starting with a sudden rise in body temperature above 40°C which may last for 2 to 6 days. Animals show a typical fever syndrome with listlessness, anorexia and loss of condition. After 1 or 2 days, dyspnea appears followed by gradual development of nervous signs. Animals are hypersensitive, have an unsteady gait and rigid movements. In typical cases, they become recumbent and exhibit pedalling movements, with muscle contractions and the head thrown back. Animals generally die during an attack. Diarrhea is common in cattle.

In peracute cases, often seen in imported breeds and especially in goats, the animals suddenly die during the hyperthermic phase without developing other symptoms. In untreated acute and peracute cases, the mortality varies from 50% to more than 90%. On the other hand only a slight hyperthermia followed by a complete recovery occurs in indigenous breeds of ruminants or in very young animals (see chapter on immunization).

In acute cases, differential diagnosis should include rabies, cerebral babesiosis (*B. bovis*) and theileriosis, tetanus, bacterial meningoencephalitis, plant poisoning and intoxication with pesticides.

At necropsy the main picture is effusion of the body cavities (Prozesky, 1987b). Hydrothorax can amount to several liters of a yellow fluid in cattle and up to 500 ml in small ruminants. Lung edema is common. Hydropericardium which is usually more pronounced in small ruminants than in cattle, has given the disease the name heartwater. A variety of inconsistent less characteristic lesions are reported, such as splenomegaly, swelling of lymph nodes, congestion of the liver, petechiae and hemorrhages in various organs. Macroscopic changes in the brain are limited to congestion of the meningeal blood vessels and occasionally edema of the meninge. In general, the longer the disease episode, the more exsudates and variety of the lesions (Uilenberg, 1983).

Microscopic lesions are non pathognomonic unless *Cowdria* colonies are found, and there are no stricking histopathological changes other than pulmonary edema and interstitial pneumonia (Prozesky, 1987b). The lesions result in an increased vascular permeability with cellular infiltrates generally containing a majority of macrophages. As a general rule, there is no correlation between the severity of the lesions and the concentration of organisms neither in ruminants nor mice (Pienaar, 1970). Moreover the limited pathological changes in infected cells indicate that *Cowdria* per se is not responsible for the increased vascular permeability (Prozesky and Du Plessis, 1985).

Definite diagnosis is usually made after death by demonstrating the presence of *Cowdria* colonies within the endothelial cells of capillaries in brain squash smears stained with Giemsa (Purchase, 1945). The staining properties of *Cowdria* are maintained 2 to 3 days at room temperature, up to 1 month in the refrigerator and at least 7 months after freezing at - 30°C (Uilenberg, 1971), making possible a confirmation of the diagnosis under field conditions.

On the live animal there is no simple method to identify the disease. Brain cortex smears can be obtained by biopsy (Synge, 1978), but although harmless for the animal if well practiced, this method is not practical for routine diagnosis. Even though *Cowdria* has been detected in leucocytes (Sahu et al., 1983), stained blood smears are not of value for use in diagnosis. *Cowdria* colonies can be seen in the cytoplasm of neutrophils harvested during the febrile phase and cultivated *in vitro* for 1 to 5 days (Sahu et al. 1983; Sahu, 1986; Logan et al., 1987). However the

technique is not reliable, since certain stocks grow rapidly, whereas others are hardly or not detectable (Jongejan et al., 1989). Isolation of the bacterium *in vitro* is difficult and takes several weeks.

The development of serological tests has long been hindered by the absence of a suitable culture system to produce large quantities of purified antigens. The first workable test was an indirect fluorescent antibody test (IFAT) using mouse peritoneal macrophages infected by the Kümm strain as antigen (Du Plessis, 1981a). Other IFAT tests were developed thereafter using infected neutrophils (Jongejan et al., 1989) or endothelial cells cultivated *in vitro* (Martinez et al., 1990).
A competitive ELISA using a monoclonal antibody to a 32 kDa protein (MAP1 protein) conserved between stocks of *Cowdria* (Jongejan and Thielemans, 1989) was developed by Jongejan et al. (1991c). Two indirect ELISA tests using semi-purified organisms as antigen (Martinez et al., 1993a) or detergent soluble antigens (Soldan et al., 1993) were also extensively used. However, all these serodiagnostic tests described previously proved to give false positive reactions with sera against several *Ehrlichia* species including *E. canis*, *E. bovis* and *E. ovina* (Du Plessis et al., 1993). Immunoblotting methods showed thereafter that the 32 kDa protein of *Cowdria* was conserved within the genus *Ehrlichia* (Jongejan et al., 1993a) and responsible for these false reactions (Mahan et al., 1993).

Two approaches were subsequently used to overcome the problem of crossreactions with *Ehrlichia*. Van Vliet et al. (1995) used an immunogenic region of the MAP1 protein (MAP1b fragment) that does not give cross-reactions with *E. bovis* and *E. ovina*, as antigen in an indirect ELISA. Cross-reactions with *E. canis* and *E. chaffeensis* are however still detected but these species are not considered to be infective for domestic ruminants. Katz et al. (1996) cloned the MAP1 gene in baculovirus and developed monoclonal antibodies to the protein for use in a competitive ELISA (Katz et al. 1997). Some crossreactions with *E. canis* and *E. chaffeensis* still exist as well as with an unclassified agent responsible for positive reactions in sera of the white-tailed deer. Both the MAP1b ELISA (Mondry et al., submitted) and the MAP1 CELISA (Mondry et al., in preparation) evaluated in the Caribbean, have shown a great improvement of the specificity compared with an ELISA using crude *Cowdria* antigens.

Molecular methods have also been developed for use in diagnostic or epidemiological studies. A cloned DNA probe was developed by Waghela et al. (1991) and used for the detection of *Cowdria* in ticks (Yunker at al., 1993) and infected sheep (Mahan et al., 1992). PCR primers were derived from the DNA sequence of the probe for use in a PCR assay (Peter et al., 1995), greatly improving the sensitivity of the *Cowdria* detection in ticks. Independantly, Kock et al. (1995) derived PCR primers from the sequence of the MAP1 gene and used them successfully to detect *Cowdria* DNA in the blood and bone marrow of wild ungulates. DNA probes and PCR assays that are very useful for research and

epidemiological studies, are nevertheless hardly applicable for routine diagnosis in Africa.

In the absence of a commercial vaccine, control of the disease can be achieved by rearing resistant animals especially in farming systems where the veterinary infrastructure is poorly developed. Hereditary factors of resistance must then be differentiated from endemic stability if improvement of animal production is based on rearing of genetically resistant animals, or on the selection of partly susceptible populations for a better resistance to the disease (Matheron et al., 1987; 1991). However, productivity of local animals is often low and upgrading by importing and crossing with exotic more productive breeds is thus necessary. The control of the disease in such breeds can be obtained by infection and treatment immunisation or natural exposure to infected ticks of young animals during the period of innate resistance (see chapter immunization procedures) and protection by colostrum (Deem et al., 1996a). Older susceptible animals can be immunised by infection and treatment. Endemic stability must thereafter be achieved by maintaining a sufficient level of natural challenge that will boost immunised adult animals or young animals infected by vertical transmission (Deem et al., 1996b), or through infected ticks during the period of innate resistance.

These methods are however hardly applicable on a large scale. They are limited to farmers with a very good technical expertise, and are nevertheless risky since significant losses have been documented.

VACCINATION AND IMMUNITY AGAINST *COWDRIA RUMINANTIUM* INFECTION

I- IMMUNIZATION PROCEDURES

Even before the causative organism was discovered, it was recognized that animals which recovered from the disease heartwater were protected to subsequent infections (Dixon, 1898; 1899a; 1899b; Alexander, 1931; Neitz, 1939).

Early attempts to transfer immunity were done by intravenous or subcutaneous inoculation of bile and other body fluids (Dixon, 1898; 1899a, 1899b), or serovaccination (Theiler, 1906), without conclusive results. Stimulation of immunity by inoculation of virulent blood or tick homogenates gave encouraging results but was regarded as dangerous (Hutcheon, 1902; Spreull, 1904). Treatment of infected animal tissues or ticks with formalin or glycerine either killed the organism making it inappropriate for immunisation, or resulted in the absence of attenuation (Alexander, 1931). Attenuation of the virulence by serial intravenous passage in sheep also failed (Du Toit and Alexander, 1931). In all these attempts, it was also noted that only animals developing clinical symptoms became immunized. Thus, immunisation procedures which seemed safe as they did not provoke adverse reactions were also always ineffective.

Already in the last century, it was established that young animals were less susceptible than older animals to intravenous inoculation with virulent blood (Dixon, 1899a). This period of non specific resistance was reported to be short-lived in lambs (7-9 days) (Neitz and Alexander, 1941; Uilenberg, 1971) and kids (2-6 weeks) (Camus, 1987; Thomas and Mansvelt, 1957) and longer in calves (3 weeks to 6 months) (Neitz and Alexander, 1941; Du Plessis et al., 1984). This age-dependant resistance was therefore considered to be independant of the immune status of the dam (Neitz and Alexander, 1941; 1945). However an important variability in susceptibility of animals both between and within breeds was reported (Neitz, 1939; Van der Merwe, 1979, Uilenberg, 1971, Du Plessis et al., 1983) and a significant percentage of animals may show a strong reaction and die even during the first days of age. Recently, vertical transmission of *Cowdria* from dams to calves was demonstrated (Deem at al., 1996a) and it was suggested that colostrum may account for the resistance of young calves (Deem et al., 1996b).

The first effective means of immunization older ruminants against heartwater known as the « infection and treatment » method, was described after the discovery of efficient chemotherapeutic agents (Neitz, 1940; Neitz and Alexander, 1941; Haig et al., 1954). Infective material is injected by the intravenous route to susceptible ruminants and the rectal temperature is monitored daily. Specific antibiotic

treatment, Uleron originally and tetracycline at present, is given as soon as the body temperature rises above 39.5°C for cattle and 40°C for small ruminants (Van der Merwe, 1987). Blood of a reacting donor animal has long been used as the source of infective material and thus refered to as blood vaccine. More recently, this blood vaccine was partly replaced in South Africa by a tick-derived vaccine consisting of homogenates of engorged *Amblyomma hebraeum* nymphs, highly infective material allowing the storage of large numbers of vaccine doses (Bezuidenhout, 1981). Despite precautions taken in the choice of donor animals and *Amblyomma* tick colonies, as well as high standard quality control of vaccine batches (Oberem and Bezuidenhout, 1987; Bezuidenhout, 1989), the transmission of blood parasites or viruses is a major risk of this type of vaccines especially when they have to be used far from their country of origin. Moreover, although allowing a good protection of animals, this method is unsuitable for mass vaccination. In effect, the infectious material should be preserved in dry ice or liquid nitrogen and thawed shortly before inoculation into animals. Fever should be monitored daily to administer the antibiotic treatment at the proper time. All injections must be given intravenously by well-trained staff. Moreover, frequent anaphylactic shocks have been reported in kids and lambs especially with the tick-vaccine.

Several strategies were proposed to simplify the vaccination procedures. Large numbers of animals can be immunised by the « block method » (Neitz and Alexander, 1945; Fick and Schuss, 1952; Poole, 1962a; 1962b). Animals are injected with vaccine and treated simultaneously and indiscriminately at a given day after inoculation, irrespective of whether they show a temperature reaction or not. Since the incubation period depends on the dose of *Cowdria* administered and possibly also on individual factors (age, breed), the use of this method needs a good standardization of the vaccine (stock used, dose). In addition, it was emphasized by Du Plessis and Malan (1987), that the more severe the reaction of the host to the immunizing infection, the stronger the immunity to subsequent challenge. Thus, there is a danger of fatal recrudescent infection if block treatment is given too early. In order to determine a more suitable day for treatment, a « controlled block method » has been developed whereby the rectal temperature of 10% of the animals is monitored daily. When indicator animals react, temperatures of all animals are taken and the reacting animals are treated (Bezuidenhout, 1989).

Recently, improvements have been proposed for the infection and treatment method. The use of endothelial cell cultures instead of blood or tick-derived material as a cheaper, more standardized way of vaccination carrying less risk of contamination by other pathogens has been developed (Brett, 1993). Freeze drying of *Cowdria* makes it possible to store the vaccine in a normal refrigerator instead of under dry-ice or liquid nitrogen (Du Plessis et al., 1990). A slow-release implant of doxycycline (Olivier et al., 1988), an antibiotic shown to be effective in the treatment of heartwater (Immelman and Dreyer, 1982), administered at the time of

infection to cattle in South Africa was claimed to avoid further monitoring. However, experiments conducted in Malawi by Lawrence et al. (1993) with these slow-release implants failed and the procedure was abandonned.

In 1991, the Senegal Stock of *Cowdria* (Jongejan et al., 1988) was attenuated by serial passages in cultures of endothelial cells (Jongejan, 1991). This stock was shown to confer a strong protection against the virulent homologous stock in small ruminants (Jongejan et al., 1993b). The same isolate was also attenuated independantly by Martinez et al. (unpublished data) who showed that the attenuation was stable after 54 passages in culture. A field trial conducted in Senegal on sheep gave good results (Gueye et al., 1994). However, the Senegal stock is poorly crossprotective with many other stocks (Jongejan et al., 1993b). Recently, the Gardel isolate originally highly pathogenic for domestic ruminants, was attenuated after more than 200 passages in culture (Martinez et al., unpublished data). The Gardel stock gives a good protection against heterologous challenge with many stocks. If the attenuation is confirmed on a greater number of animals, the association of both Gardel and Senegal attenuated stocks could lead to the formulation of a wide spectrum live vaccine against heartwater.

II- IMMUNITY TO *COWDRIA RUMINANTIUM INFECTION*

Duration of immunity and the persistence of Cowdria in animal tissues

Numerous observations have shown that immunity develops in animals that have recovered from either natural or experimental infection. In addition, the question of whether immunity in heartwater is sterile or associated with the persistence of *Cowdria* has often been raised.

In ruminants

The duration of immunity reported in sheep varies between 6 months and 5 years but was generally long lasting (Neitz, 1939; 1968; Spreull, 1922; Henning, 1956; Haig, 1955; Stewart, 1987a;, Alexander et al., 1946).

In cattle, protection of at least 2 or 3 years was reported by several authors (Du Plessis and Bezuidenhout, 1979; Neitz and Alexander, 1945; Du Plessis et al., 1992b). In contrast, Du Plessis et al. (1984) found that calves were only partially protected when challenged 6 months after immunisation.

The duration of immunity in goats is less well documented. Several breeds are regarded as very susceptible to heartwater and may suffer losses after infection and treatment (Yunker, 1996). Creole goats from Guadeloupe were resistant to

homologous challenge for at least 2 years (Camus, 1989). Immunity in Angora goats may be incomplete 107 to 205 days after vaccination according to Du Plessis et al. (1983) but Gruss (1987) was able to protect Angora goats for at least 1 year without losses due to immunization. Despite differences observed by different groups, it appears that in most cases, a strong and long lasting protection can be achieved by the traditional methods of immunization, and that discrepancies are likely to be due to badly controlled factors as the virulent dose inoculated to animals and treatment not given at the optimum time as emphasized by Du Plessis et al. (1983).

Until 1989, all studies indicated the development of a sterile immunity in ruminants. The circulation of the pathogen in the blood stream as detected by subinoculation of blood (Alexander, 1931; Ilemobade, 1978) and tissue extracts (Neitz, 1939) into susceptible ruminants never exceeded 2 months, and in most cases was even limited to 2-3 weeks. Blood appeared to be infective for *Amblyomma variegatum* larvae for only 8 days following a primary infection of goats (Barré and Camus, 1987). Immune sheep treated with dithiosemicarbazone and therefore sterilized from *Cowdria* infection, are more susceptible to challenge than non-treated sheep, supporting the view that immunity is linked to the persistence of the pathogen in the tissues (Du Plessis, 1981b). The splenectomy of recovered animals also does not result in a relapse of the disease as it does for many other blood protozoal or rickettsial diseases (Neitz, 1939).

Recently, it was shown by Andrew and Norval (1989) that cattle, sheep and African buffalo may remain intermittently infective for *Amblyomma hebraeum* nymphs for at least 8 months after infection. A carrier state of at least 11 months was thereafter confirmed in goats (Camus, 1992). The presence of *Cowdria* was recently demonstrated by PCR in blood and bone marrow of clinically normal wild ungulates indicating that this organism can also persist in wildlife (Kock et al., 1995). Supporting the idea that the persistence of *Cowdria* in recovered ruminants is a common feature of cowdriosis despite the difficulty to reveal this presence, are relapses of heartwater observed when animals are submitted to stress such as vaccinations, dipping or transportation (Uilenberg, 1983). Tick toxicosis has also been reported by Neitz (1968) to induce relapse of cowdriosis in recovered animals.

In mice

Mice are able to develop a specific immunity to *Cowdria* as demonstrated by infection with various mice stocks followed by treatment with oxytetracyclin or dithiosemicarbazone (Stewart, 1987b). Several stocks have been found pathogenic or infective but not pathogenic to mice (review by MacKenzie and McHardy, 1987; Du Plessis et al., 1989).

In the case of the Kümm stock (Du Plessis and Kümm, 1971), the duration of immunity is at least 18 months with persistence of the organisms in the liver, spleen and lung (Du Plessis, 1982). The Welgevonden stock (Du Plessis, 1985) was demonstrated to persist in the tissues of mice 2 months after infection but not 4 months thereafter, while immunity lasted for 11 months (Du Plessis et al., 1991). The presence of the Kwanyanga stock (MacKenzie and Van Rooyen, 1981), could not be demonstrated in mice 4 months after infection although the animals were still protected (Wassink et al., 1987). In addition, mice infected and sterilised with dithiosemicarbazone resist challenge indicating that a sterile immunity may occur (Wassink et al., 1987). In contrast, the Senegal stock was shown to persist for a year in organs of Swiss mice, but it was reported to be non pathogenic (Wassink et al., 1990). But cell culture material of the Senegal stock is highly pathogenic to outbred mice (Martinez, unpublished data). Apparent differences of pathogenicity are possibly due to dose effects and differences in infectious titres between cell culture supernatants and blood stabilates.

Although the persistence of *Cowdria* in the susceptible vertebrate host seems to be a common feature, the duration of the carrier states reported is variable. This can be due to the lack of sensitivity of the methods used to reveal the presence of the parasite in the tissues, but also to a variable degree of coevolution between the host (at the individual level) and the parasite (at the stock level), the host being able to mount a proper immune response and *Cowdria* to escape a deleterious immune response without killing its host. However, there is evidence that the duration of immunity is not correlated with the duration of the carrier state. Recently, several authors (Martinez et al., 1994; 1996; Mahan et al., 1995; Totté et al., 1997) have successfully immunised ruminants with inactivated elementary bodies of *Cowdria*, showing that live *Cowdria* is not necessary for protection.

Role of antibodies and complement in protection

Antibodies do not seem to play a major role in immunity to cowdriosis. Several authors failed to protect ruminants or even modify the course of the disease by transfer of immune serum or gamma-globulins purified from immune animals whether given before, after or simultaneously with infective blood (Alexander, 1931; Du Plessis, 1970; 1982). The protection or the intensity of the clinical reaction of infected animals is not correlated with the antibody titres (Du Plessis, 1984). However, Byrom et al. (1993) and Du Plessis (1993) were able to demonstrate some degree of neutralisation *in vitro*.

Du Plessis (1993) reported that an homologous immune serum significantly inhibited the infectivity of the Kümm stock of *Cowdria* for outbred Swiss mice under specific

experimental conditions. Except for a Ball 3 sheep antiserum, the infectivity was not affected by the use of immune sheep sera raised against the heterologous stocks Mali, Welgevonden and Kwanyanga. These results are in agreement with the absence of cross-protection between the Kümm stocks and these 3 stocks. However the inhibitory effect of the Ball 3 serum was unexpected (Du Plessis & van Gas, 1989; Logan et al., 1987). The presence of rabbit complement was essential for this seroneutralisation suggesting that inhibition of *Cowdria* infectivity by sheep immune serum can be attributed to a direct lytic effect through the activation of the antibody-dependant complement pathway. The results of these experiments differ from an earlier finding by the same author (Du Plessis et al., 1991) whereby 4 sheep antisera with high IFAT antibody titres against 4 stocks of *Cowdria* had no inhibitory effect on the infectivity of the Kümm stock for mice in presence of complement. The anti-Kümm immune serum had an inhibitory effect on the infectivity of *Cowdria* only when it was incubated with macrophages from immune or non immune mice, the *Cowdria* and complement, thus suggesting the importance of opsonization. The only differences between both studies were the concentrations of complement and *Cowdria*. It cannot be excluded that the peritoneal macrophages of the mice injected with the inoculum may have facilitated their survival in the first study (Du Plessis, 1993). Whether the inhibition of *Cowdria* infectivity can be attributed to an opsonic effect or to a direct lytic effect through the activation of the antibody-dependant complement pathway remains unclear.

Byrom et al. (1993) showed that hyperimmune serum from DBA/2 and Balb/c mice as well as 2 bovine sera (1 from an experimentally infected animal and 1 from an animal infected in the field) had a strong neutralizing effect on the infection of endothelial cells *in vitro*. These authors were unable to confer protection to DBA/2 or Balb/c mice against an intravenous challenge of *Cowdria* by transfer of the sera which were neutralizing *in vitro*. Addition of complement had no beneficial effect.

Thus, despite the conflicting results of neutralization tests *in vitro* and the failure to transfer protection *in vivo*, a certain role of antibodies cannot be completely excluded. In particular, the necessity of optimal conditions to obtain any detectable effect has been emphasized by Du Plessis (1993) and could account for the discrepancies between experiments.

Cellular immunity and role of cytokines in protection

Since *Cowdria* is an obligate intracellular parasite, it is a common belief that immunity to heartwater is cell mediated. As a matter of fact, in a mouse model of cowdriosis, Du Plessis (1982) showed that the transfer of immune spleen cells protected naive syngenic DBA2 mice against a challenge with the Kümm stock given 30 and 60 days later. Athymic nude mice proved to be unable to recover from or

mount an immune response against infection with either the Kümm or the Welgevonden stocks of *Cowdria*, even after treatment (Du Plessis et al., 1991). In the same study, adoptive transfer of immune spleen cells rendered 3/7 mice resistant to challenge and retarded the mortality of those that died. Moreover, immune spleen cells depleted of Lyt-2^+ T-cells ($CD8^+$ cells) were unable to confer resistance to challenge with *C. ruminantium* to recipient mice, whereas the depletion of $L_3T_4^+$ ($CD4^+$ cells) had no effect on this protection. These observations constitute supportive evidence that the immunity in heartwater is T-cell mediated, and suggest the importance of $CD8^+$ cell-mediated cytotoxicity mechanisms as described in other diseases due to obligate intracellular microorganisms. However, these authors were unable to affect the course of the infection or the resistance of immune mice to challenge, by *in vivo* depletion of Lyt-2^+ lymphocytes using monoclonal antibodies.

Flow cytometric analysis of the peripheral T cell populations in mice immunised by infection and treatment with the Welgevonden stock, followed by 2 reinfections without treatment at day 23 and 50 post infection (p.i.), has been performed by Du Plessis et al. (1992a). A depletion of the percentage of $CD4^+$ lymphocytes from 41% to 24% was observed at day 20 p.i., followed by a return to preinfection values around day 60 p.i. Percentages of $CD8^+$ cells ranging from 3.9% and 5.9% until day 20 p.i., increased more than 3 fold after the first reinfection and remained at high level until the end of the experiment. This observation concurs with the earlier finding that immunity in heartwater is largely mediated by $CD8^+$ T lymphocytes.

Assuming that immunity to cowdriosis is mainly T-cell mediated, cytokines are expected to play an important role. Interferon-γ (IFNγ) plays a crucial role in the clearance of the pathogen in many rickettsial infections (Turco and Winkler, 1984; Palmer et al., 1984; Hanson, 1991a; 1991b; Park and Rikihisa, 1992). As a matter of fact, Totté et al. (1993; 1996), were able to completely inhibit the growth of *Cowdria* in infected bovine umbilical endothelial cells (BUEC), bovine microvessels endothelial cells (BMEC), caprine jugular endothelial cells (CJEC), and human umbilical vein endothelial cells (HUVEC) *in vitro*, with 0.5 U/ml of recombinant bovine INFγ added 24 hours after infection. *Cowdria* appeared to be very sensitive to INF-γ compared to other intracellular microrganisms (Shemer and Sarov, 1985). Such an inhibition was also obtained with 1% or 2% of concanavalin A-stimulated bovine lymphocytes supernatants by Mahan et al. (1994). IFNγ which is secreted in large quantity by mitogen-stimulated T-cells was identified as the inhibiting cytokine since the anti-*Cowdria* activity was completely abolished by incubation with an anti-INFγ monoclonal antibody, whereas an anti-tumor necrosis factor-α (TNFα) monoclonal antibody had no effect (Mahan et al., 1996). In both studies, inhibition of *Cowdria* growth was obtained irrespective of the *Cowdria* stock, or the origin of the cells. Protection of mice against *Cowdria* (Welgevonden stock) infection was also achieved by daily intraperitoneal injection of INFγ during 2 weeks of incubation

period, whereas injection of TNFα exerted no effect (Totté et al., 1994c). These authors were unable to show any effect of the injection of Anti-IL-6, anti-TNFα and surprisingly even anti- INFγ neutralising antibodies.

There is evidence that interferons α (IFNα) and β (IFNβ) can inhibit to various extents, rickettsial replication in the host cells (Turco and Winkler, 1990; Hanson, 1991b). INFα was found to significantly reduce the yield of *Cowdria* organisms in BME cells and was found to be 10,000 times less efficient than INFγ (Totté et al., 1993; 1994a). Interestingly, in contrast to BMEC, BUEC were not sensitive to the anti-*Cowdria* effect of INFα. This difference in sensitivity between endothelial cells from large vessels and from capillaries if confirmed, could be relevant to the immunopathology of cowdriosis. A correlation was also found *in vivo* between survival to *Cowdria* infection and the presence of a significant level of INFα (*in vivo* levels between 80 and 100 U/ml) in the plasma of infected cattle (Totté et al., 1989; 1994a). Such amounts of INFα partially inhibit the development of *Cowdria* in endothelial cells *in vitro*. Since the complete inhibition of *Cowdria in vitro* could never be achieved even at very high concentrations (up to 1000 U/ml), it was suggested by these authors that INFα produced *in vivo* in response to *Cowdria* infection, slows down the course of the infection and thus allow the animal to mount a protective immune response. INFα which is known to play an important role in T cell differenciation towards a Th1-type of immune response (Belardelli and Gresser, 1996), could be more widely involved in protection against *Cowdria* than only by a direct effect on growth.

Other molecules are nevertheless involved in immunity. Recombinant myeloperoxydase, an enzymatic component of the defense mechanism of neutrophils and monocytes, injected at day 1, 2, 3 and 4 p.i. appeared to confer protection to mice against a lethal challenge (Totté et al., 1994b).

III- THE DIVERSITY OF *COWDRIA*

Already in1931, Alexander stated that plurality in infectious agents must be accepted. In the case of heartwater the number of different strains appears to be great. Alexander based his conclusions on cross-immunity tests conducted with a large number of strains, but no further details were given. Relapse of heartwater on immunised animals under field conditions was also ascribed to reinfection with an antigenically different strain of *Cowdria*, although Neitz (1939) was of the opinion that these reactions were likely to be due to a partial or complete loss of immunity. This author found no differences between several South African strains in cross-immunity experiments in sheep. Mild temperature reactions after heterologous

challenge were attributed to a partial loss of immunity (Neitz, 1939; Neitz et al., 1947).

So far, there is no simple and reliable method for typing *Cowdria* stocks. Although Jongejan et al. (1989) described the existence of serotypes when comparing 5 stocks by immunofluorescence, Du Plessis at al. (1989) were unable to correlate the antigenic diversity of *Cowdria* stocks to antibodies detected by IFAT. Martinez et al. (1990) also did not identify true serotypes by IFAT, despite differences observed in the titre of sera tested on different *Cowdria* antigens. Recently, sequence heterogeneity of the MAP1 gene, first cloned by Van Vliet et al. (1994), was described (Reddy et al., 1995). However, MAP1 is a highly conserved protein in the genera *Cowdria* and *Ehrlichia* (Jongejan et al., 1993a), that does not confer protection against a virulent challenge (Van Kleef et al., 1993) and will therefore be of little help in grouping stocks according to their immunoprotective capacity.

More recently, a series of cross-immunity studies were conducted by several groups to address the question of the antigenic diversity of *Cowdria*. For a long time, it was thought that only one antigenic type of *Cowdria* affected ruminants. Stocks from widely distant countries such as Nigeria (Nigeria stock), South Africa (Ball 3, Zeerust), Sudan (Umm Banein stock), Sao Tomé (Sao Tomé stock), and Guadeloupe in the Caribbean (Gardel stock) proved to be crossprotective in goats (Van Winkelhoff and Uilenberg, 1981; Uilenberg et al., 1983; Uilenberg et al., 1985; Brown et al., 1989). Alternatively, several stocks from South-Africa (Kümm, Kwanyanga, Nonile and Welgevonden stocks), which were found to be pathogenic to mice in contrast to the first isolates tested, showed no or only limited cross-immunity between themselves, with the reference Ball 3 stock, and all other stocks tested isolated outside South Africa (Du Plessis and Kümm, 1971; Du Plessis, 1981a; Du Plessis, 1985; MacKenzie and Van Rooyen, 1981; MacKenzie and McHardy, 1987; Uilenberg et al., 1985; Jongejan et al., 1991b). It is now known that this lack of cross-protection is extensive, irrespective of pathogenicity to mice (Uilenberg, 1996). It may be responsible for breakdowns of immunity in the fields, especially in highly susceptible goats which express partial cross-immunity differences more apparently than sheep and cattle (Jongejan et al., 1991b). Challenge of seropositive small stocks in heartwater endemic areas of South Africa with the Ball 3 and the Welgevonden stocks revealed that between 19% and 32% of animals were still susceptible to challenge (Du Plessis and Van Gas, 1989). From these observations, it is tempting to conclude that a combination of several stocks could help to overcome the problem of antigenic diversity. However, a first study by Du Plessis et al. (1990) using different combinations of the Ball 3 (Haig, 1952), Kwanyanga (MacKenzie and Van Rooyen, 1981), and Mara (Du Plessis et al., 1989) stocks did not significantly improve the efficacy of immune protection compared with vaccination with a single stock.

As a result of these studies, it is now known that cross-protection between stocks can be complete, partial or absent, unilateral or bilateral (Uilenberg and Camus, 1993). So far, as already stated by Alexander (1931), no master isolate conferring a complete protection has been isolated. Therefore, there is a need to continue with the characterization of isolates, and to quantify the extent of the diversity in terms of cross-protection in order to develop proper vaccines. This implies the standardization of cross-immunity procedures to avoid discrepancies or variations in the results leading to erroneous conclusions. Since the detection of antigenic differences is more sensitive in goats than in other ruminant species (Jongejan et al., 1991b), the goat is the animal of choice for cross-immunity trials. As the incubation period and the fate of the disease is affected by the infectivity of the dose, it is also essential to conduct cross-immunity trials with titrated and standardized inocula. This is also the only way to compare the virulence of different stocks of *Cowdria*.

REFERENCES

1. Alexander, R.A., 1931. Heartwater. The present state of our knowledge of the disease. 17th Report of the Director of Veterinary Services and Animal Industry, Union of South Africa, 89-150.

2. Alexander, R.A., Neitz, W.O., Adelaar, T.F., 1946. Heartwater. Farming in South Africa, 21, 548-552.

3. Allsopp, B. A., Allsop, M.T., DU Plessis, J.H., 1996. Uncharacterized *Ehrlichia* spp. may contribute to clinical heartwater. *Ann. N. Y. Acad. Sci.* 791, 17-24.

4. Andrew, H.R., Norval, R.A.I., 1989. The carrier status of sheep, cattle and African buffalo recovered from heartwater. *Vet. Parasitol.* 34, 261-266.

5. Barré, N., Camus E., 1987. The reservoir status of goats recovered from heartwater. *Onderstepoort J. vet. Res.* 54, 435-437.

6. Barré, N., Uilenberg, G., Morel, P.C., Campus, E., 1987. Danger of introducing heartwater onto the american mainland : potential role of indigenous and exotic Amblyomma ticks. *Onderstepoort J. vet. Res.* 54, 406-417.

7. Belardelli, F., Gresser, I., 1996. The neglected role of type I interferon in the T-cell response: implications for its clinical use. *Immunol. Today* 17, 369-372.

8. Bezuidenhout, J.D., 1981. The development of a new heartwater vaccine, using *Amblyomma hebraeum* nymphae infected with *Cowdria ruminantium*. Proceedings of the International Conference "Tick biology and control", Grahamstown, South Africa, 41-45.

9. Bezuidenhout, J.D. 1987. Natural transmission of heartwater. *Onderstepoort J. Vet. Res.* 54, 349-351.

10. Bezuidenhout, J.D. 1988. Sekere aspekte van hartwateroordraging, voorkoms van die organisme in bosluise en *in vitro* kweking. Doctor of Science Thesis, University of Pretoria.

11. Bezuidenhout, J.D., 1989. *Cowdria* vaccines. In Wright I.G. editor. Veterinary protozoan and hemoparasite vaccines. Boca Raton, CRC Press, Inc., pp 31-42.

12. Bezuidenhout, J.D., Jacobsz, C.J., 1986. Proof of transovarial transmmission of *Cowdria ruminantium* by *Amblyomma hebraeum*. *Onderstepoort J. Vet. Res.* 53, 31-34.

13. Bezuidenhout, J.D., Paterson, C.L., Barnard, B.J.H., 1985. *In vitro* cultivation of *Cowdria ruminantium*.. *Onderstepoort J. vet. Res.* 51, 133-120.

14. Birnie, E.F., Burridge, M.J., Camus, E., Barré, N., 1984. Heartwater in the Caribbean : isolation of *Cowdria ruminantium* from Antigua. *Vet. Rec.* 116, 121-123.

15. Brett, S., J.D. Bezuidenhout, De Waal, D.T., 1992. The establishment of an ovine cerebral endothelial cell line (SBE189) that supports the growth of *Cowdria ruminantium*. *Jl. S. Afr. vet. Assoc.* 63, 87.

16. Brett, S., 1993. Heartwater tissue culture vaccine. In Biennal report of the Veterinary Research Institute Onderstepoort, 1990-1991: 19. Department of Agricultural Development. Pretoria, South Africa.

17. Brown, C.C., Logan, L.L., Mebus, C.A., Nagorski, K., 1989. Protection of goats against Caribbean and African heartwater isolates by the Ball 3 heartwater vaccine. *Trop. Anim. Hlth Prod.*, 21, 100-106.

18. Byrom, B., Yunker, C.E., 1990. Improved culture conditions for *Cowdria ruminantium* (Rickettsiales) the agent of heartwater disease of domestic ruminants. *Cytotechnology*, 4, 285-290.

19. Byrom, B., Mahan, S.M., Barbet, A.F., 1993. The development of antibody to *Cowdria ruminantium* in mice and its role in heartwater disease. *Rev. Elev. Méd. Vét. Pays trop.* 46, 197-201.

20. Camus, E., 1987. Contribution à l'étude épidémiologique de la cowdriose (*Cowdria ruminantium*) en Guadeloupe. Thèse de Doctorat es-Sciences, Université de Paris-Sud. Orsay, 202 p.

21. Camus, E., 1989. Etude épidémiologique de la cowdriose à *Cowdria ruminantium* en Guadeloupe. Etudes et synthèses de l'IEMVT, No. 33. IEMVT, Maisons-Alfort, 196 pp.

22. Camus, E., 1992. Le portage asymptomatique de bovins et chèvres créoles guéris de la cowdriose en Guadeloupe. *Rev. Elev. Med. vet. Pays trop.* 45, 133-135.

23. Camus, E., N. Barré. 1992. The role of *Amblyomma variegatum* in the transmission of heartwater with special references to Guadeloupe: current issues and perspectives. *Ann. N.Y. Acad. Sci.* 653, 33-41.

24. Camus, E., Barre, N., Martinez, D., Uilenberg, G., 1996. Heartwater (cowdriosis) : a review. OIE, Paris, pp 177.

25. Cowdry, E.V., 1925a. Studies on the aetiology of heartwater : II. *Rickettsia ruminantium* (Northern Species) in the tissues of infected animals., *J. Exp. Med.* 42, 231-252.

26. Cowdry, E.V., 1925b. Studies on the aetiology of heartwater : II. *Rickettsia ruminantium* (Northern Species) in the tissues of ticks transmitting the disease. *J. Exp. Med.*, 42, 253-274.

27. Dame, J.B., S.M. Mahan, and C.A. Yowell. 1992. Phylogenetic relationship of *Cowdria ruminantium*, agent of heartwater, to *Anaplasma marginale* and other members of the order *Rickettsiales* determined on the basis of 16S rRNA sequence. *Int. J. Syst. Bacteriol.* 42, 270-274.

28. Deem, S.L., Donachie, P.L., Norval, R.A.I., 1996a. Colostrum from dams living in a heartwater-endemic area influences calfhood immunity to *Cowdria ruminantium*. *Vet. Parasitol.* 61, 133-144.

29. Deem, S.L., Norval, R.A.I., Donachie, P.L., Mahan, S.M., 1996b. Demonstration of vertical transmission of *Cowdria ruminantium*, the causative agent of heartwater, from cows to their calves. *Vet. Parasitol.* 61, 119-132.

30. Dixon, R.W., 1898. Heartwater experiments. *Agric. J. Cape of Good Hope,* 12, 754-762.

31. Dixon, R.W., 1899a. Heartwater experiments. *Agric. J. Cape of Good Hope*, 14, 205-207.

32. Dixon, R.W., 1899b. Heartwater experiments. *Agric. J. Cape of Good Hope*, 15, 790-792.

33. Du Plessis, J.L., 1970. Immunity in heartwater : I. A preliminary note on the role of serum antibodies. *Onderstepoort J. Vet. Res.* 37, 3, 147-150.

34. Du Plessis, J. L. 1981a. The application of the indirect fluorescent antibody test to the serology of heartwater. in: Proceedings of an International Congress on tick biology and control, Rhodes University, Grahamstown, South Africa. 1981. p. 47-52.

35. Du Plessis, J.L., 1981b. The influence of dithiosemicarbazone on the immunity of sheep to hearwater. *Onderstepoort J. vet. Res.* 48, 175-176.

36. Du Plessis, J.L., 1982. Mice infected with a *Cowdria ruminantium*-like agent as a model in the study of heartwater. Thesis, Veterinary Science, University of Pretoria, South Africa.

37. Du Plessis, J.L., 1984. Colostrum-derived antibodies to *Cowdria ruminantium* in the serum of calves and lambs. *Onderstepoort J. vet. Res.* 51, 275-276.

38. Du Plessis, J.L., 1985. A method for determining the *Cowdria ruminantium* infection rate of *Amblyomma hebraeum* : effects in mice injected with tick homogenate. *Onderstepoort J. vet. Res.* 52, 55-61.

39. Du Plessis, J.L., 1993. An *in vitro* test to demonstrate the inhibitory effect of homologous immune serum on the infectivity of *Cowdria ruminantium*. *Onderstepoort J. vet. Res.* 60, 69-73.

40. Du Plessis, J.L., Bezuidenhout, J.D., 1979. Investigation on the natural and acquired resistance of cattle to artificial infection with *Cowdria ruminantium*. *J. S. Afr. Vet. Assoc.* 50, 334-338.

41. Du Plessis, J.L., Kumm, N.A.L., 1971. The passage of *Cowdria ruminantium* in mice. *J. S. Afr. Vet. Med. Assoc.* 42, 217-221.

42. Du Plessis, J.L., Malan, L., 1987. The block method of vaccination against heartwater. *Onderstepoort J. vet. Res.* 54, 493-495.

43. Du Plessis, J.L., Van Gas, L., 1989. Immunity of tick-exposed seronegative and seropositive small stock challenged with two stocks of *Cowdria ruminantium*. *Onderstepoort J. vet. Res.* 56, 185-188.

44. Du Plessis, J.L., Jansen, B.C., Prozesky, L., 1983. Heartwater in Angora goats. I- Immunity subsequent to artificial infection and treatment. *Onderstepoort J. vet. Res.* 50, 137-143.

45. Du Plessis, J.L., Bezuidenhout, J.D., Ludemann, C.J.F., 1984. The immunization of calves against heartwater : subsequent immunity both in the absence and presence of natural tick challenge. *Onderstepoort J. vet. Res.* 15, 193-196.

46. Du Plessis, J.L., Camus E., Oberem, P.T., Malan, L.T., 1987. Heartwater serology : some problems with the interpretation of results. *Onderstepoort J. vet. Res.* 54, 327-329.

47. Du Plessis, J.L., Van Gas, L., Olivier, J.A., Bezuidenhout, J.D., 1989. The heterogenicity of *Cowdria ruminantium* stocks : cross- immunity and serology in sheep and pathogenicity to mice. *Onderstepoort J. vet. Res.* 56, 195-201.

48. Du Plessis , J.L., Potgieter, T., Van Gas, L., 1990. An attempt to improve the immunization of sheep against heartwater by using different combinations of stocks of *Cowdria ruminantium*. *Onderstepoort J. vet. Res.* 57, 205-208.

49. Du Plessis, J.L., Van Gas, L., Labuschagne, F.J., Wijma, S., 1990. The freeze-drying of *Cowdria ruminantium*. *Onderstepoort J. vet. Res.* 57, 145-149.

50. Du Plessis, J.L., Berche, P., Van Gas, L., 1991. T cell-mediated immunity to *Cowdria ruminantium* in mice : the protective role of Lyt-2+ T cells. *Onderstepoort J. vet. Res.* 58, 171-179.

51. Du Plessis, J.L., Gray, C., Van Strijp M.F., 1992a. Flow cytometric analysis of T cell response in mice infected with *Cowdria ruminantium*. *Onderstepoort J. vet. Res.* 59, 337-338.

52. Du Plessis, J.L., Loock P.J., Ludemann C.J.F. 1992b. Adult *Amblyomma hebraeum* burdens and heartwater endemic stability in cattle. *Onderstepoort J. vet. Res.* 59, 75-89.

53. Du Plessis, J.L., Bezuidenhout, J.D., Brett, M.S., Camus, E., Jongejan, F., Mahan, S.M., Martinez, D., 1993. The serodiagnosis of heartwater : a comparison of five tests. *Rev. Elev. Méd. Vét. Pays trop.* 46, 123-129.

54. Du Toit, P.J., Alexander, R.A., 1931. An attempt to attenuate the virus of heartwater by passage. 17th Report of the Director of Veterinary Services and Animal Industry, 1, 151-160.

55. Fick, J.F., Schuss, J., 1952. Heartwater immunisation under field conditions in Swaziland. *J. S.Afr. Vet. Med. Assoc.* 23, 1, 9-14.

56. Flach, E.J., Woodford, J.D., Morzaria, S.P., Dolan, T.T., Shambwana, I., 1990. Identification of *Babesia bovis* and *Cowdria ruminantium* on the island of Unguya, Zanzibar. *Vet. Rec.* 126, 57-59.

57. Gruss, B., 1987. Problems encountered in the control of heartwater in Angora goats. *Onderstepoort J. vet. Res.* 54, 513-515.

58. Gueye, A., Mbengue, Mb., Kebe, B., Diouf, A., 1982. Note epizootiologique sur la cowdriose bovine dans les Niayes au Sénégal. *Rev. Elev. Med. vet. Pays trop.* 35, 212-219.

59. Gueye, A., Jongejan, F., Mbengue, Mb., Diouf, A., Uilenberg, G., 1994. Essai sur le terrain d'un vaccin atténué contre la cowdriose. *Rev. Elev. Med. vet. Pays trop.* 47, 401-404.

60. Haig, D.A., 1952. Note on the use of the white mouse for the transport of strains of heartwater. *J. S. Afr. Vet. Med. Assoc.* 23.

61. Haig, D.A., 1955. Tick-borne Rickettsioses in South Africa. *Adv. Vet. Sci.* 2, 307-325.

62. Haig, D.A., Alexander, R.A., Weiss, K.E., 1954. Treatment of heartwater with Terramycin. *J. S. Afr. Vet. Med. Assoc.* 25, 45-48.

63. Hanson, B., 1991a. Susceptibility of *Rickettsia tsutsugamushi* Gilliam to gamma interferon in cultured mouse cells. *Infect. Immun.* 59, 4125-4133.

64. Hanson, B., 1991b. Comparative susceptibility to mouse interferons of *Rickettsia tsutsugamushi* strains with different virulence in mice and of *Rickettsia rickettsii*. *Infect. Immun.* 59, 4134-4141.

65. Henning, M.W., 1956. Animal diseases in South Africa. Third edition, Onderstepoort, South African Central News Agency, Ltd, 1115-1179.

66. Holland, C.J., Logan, L.L., Mebus, C.A., Ristic, M., 1987. The serological relationship between *Cowdria ruminantium* and certain members of the genus *Ehrlichia*. *Onderstepoort J. vet. Res.* 54, 331.

67. Hutcheon, D., 1902. Heartwater in sheep and goats. *Agric. J. Cape of Good Hope*, 20, 633-641.

68. Ilemobade, A.A., 1978. The persistence of *Cowdria ruminantium* in the blood of recovered animals. *Trop. Anim. Hlth. Prod.* 10, 170.

69. Immelman, A., Dreyer, G., 1982. The use of doxycycline to control heartwater in sheep. *Jl S. Afr. Vet. Assoc.* 53, 23-24.

70. Jongejan, F., 1991. Protective immunity to hearwater *(Cowdria ruminantium* infection) is acquired after vaccination with in vitro-attenuated Rickettsiae. *Infect. Immun.* 59, 729-731.

71. Jongejan, F. 1992a. Studies on the transmission of *Cowdria ruminantium* by African and American *Amblyomma* ticks. *In* Proceedings and Abstracts of the first International Conference on tick-borne pathogens at the host vector interface : an agenda for research (U.G. Munderloh & T.J. Kurtti, Eds), St Paul, Minnesota, 15-18 seprember, 143.

72. Jongejan, F. 1992b. Experimental transmission of *Cowdria ruminantium (Rickettsiales)* by the American reptile tick *Amblyomma dissimile* Koch, 1844. *Exp. Appl. Acarol.* 15, 117-121.

73. Jongejan, F., Thielemans, M.J.C., 1989. Identification of an immunodominant antigenically conserved 32-kilodalton protein from *Cowdria ruminantium. Infect. Immun.* 57, 3243-3246.

74. Jongejan, F., Uilenberg, G., Franssen, F.J., Gueye, A., Nieuwenhuijs J., 1988. Antigenic differences between stocks of *Cowdria ruminantium. Res. Vet. Sci.* 44, 186-189.

75. Jongejan, F., L.A. Wassink, M.J.C. Thielemans, N.M. Perie, Uilenberg, G., 1989. Serotypes in *Cowdria ruminantium* and their relationship with *Ehrlichia phagocytophila* determined by immunofluorescence. *Vet. Microbiol.* 21, 31-40.

76. Jongejan, F., R. Bax, M.J. Meddens, and W.G. Quint. 1991a. *Cowdria ruminantium* is recognized by a monoclonal antibody directed against the major outer membrane protein of *Chlamydia trachomatis. Vet. Microbiol.* 27, 115-123.

77. Jongejan, F., Thielemans, M.J.C., Briere, C., Uilenberg, G., 1991b. Antigenic diversity of *Cowdria ruminantium* isolates determined by cross-immunity. *Res. Vet. Sci.* 51, 24-28.

78. Jongejan, F., Thielemans, M.J.C. de Groot, M., van Kooten, P.J., van der Zeijst, B.A.M., 1991c. Competitive enzyme linked immunosorbent assay for heartwater using monoclonal antibodies to a *Cowdria ruminantium*- specific 32-kilodalton protein. *Vet. Microbiol.* 28, 199-211.

79. Jongejan, F., Zandbergen, T.A., van de Wiel, P.A., De Groot, M., Uilenberg, G., 1991d. The tick-borne rickettsia *Cowdria ruminantium* has a *Chlamydia*-like developmental cycle. *Onderstepoort J. Vet. Res.* 58, 227-237.

80. Jongejan, F., de Vries, N., Nieuwenhuijs, J., Wassink, L. A., van Vliet., A. H. M., 1993a. The immunodominant 32-kilodalton protein of *Cowdria ruminantium* is conserved within the genus *Ehrlichia*. *Rev. Élev. Méd. vét. Trop.* 46, 145-152.

81. Jongejan, F., Vogel, S., Gueye, A., Uilenberg, G., 1993b. Vaccination against heartwater using *in vitro* attenuated *Cowdria ruminantium* organisms. *Rev. Elev. Med. vet. Pays trop.* 46, 223-227.

82. Katz, J. B., Barbet, A. F., Mahan, S. M., Kumbula, D., Lockhart, J. M., Keel, M. K., Dawson, J. E., Olson, J. G., Ewing, S.A., 1996. A recombinant antigen from the heartwater agent (*C. ruminantium*) reactive with antibodies in some southeastern United States white-tailed deer (*Odocoileus virginianus*), but not cattle sera. *J. Wildl. Dis.* 32, 424-430.

83. Katz, J. B., Dewald, R., Dawson, J.E., Camus, E., Martinez, D., Mondry, R., 1997. Development and evaluation of a recombinant antigen, monoclonal antibody-based competitive ELISA for heartwater serodiagnosis. *J. Vet. Diagn. Invest.* (in press)

84. Kocan, K.M., Bezuidenhout, J.D., 1987. Morphology and development of *Cowdria ruminantium* in *Amblyomma hebraeum*. Onderstepoort *J. Vet. Res.* 54, 177-182.

85. Kocan, K.M., Norval, R.A.I., Donovan, P.L., 1993. Development and transmission of *Cowdria ruminantium* by *Amblyomma* males transferred from infected to susceptible sheep. *Rev. Elev. Méd. vét. Pays trop.* 46, 183-188..

86. Kock, N.D., Van Vliet, A.H.M., Charlton, K., Jongejan, F., 1995. Detection of *Cowdria ruminantium* in blood and bone marrow samples from clinically normal, free-ranging Zimbabwean wild ungulates. *J. Clin. Microbiol.* 33, 2501-2504.

87. Lawrence, J.A., Tjornehoj, K., Whiteland, A.P., Kafuwa, P.T., 1993. Doxycycline implants inhibit immunity to heartwater in zebu-cross cattle after vaccination. *ECF-ABH Newsletter*, 8, 2-3.

88. Logan, L.L., Birnie, E.F., Mebus, L.A., 1987. Cross-immuniy between isolates of *Cowdria ruminantium*. *Onderstepoort J. vet. Res.* 54, 345.

89. Logan, L.L., Whyard, T.L., Quintero, J.C., Mebus, C.A., 1987. The development of *Cowdria ruminantium* in neutrophils. *Onderstepoort J. vet. Res.* 54, 197-204.

90. Logan, L.L., Tembel, Y.S., Miller, O.K., 1988. The importance of heartwater *(Cowdria ruminantium)* in Mali. *Bull. Anim. Hlth Prod. Afr.* 36, 89-92.

91. Mackenzie, P.K.I., MacHardy, N., 1987. *Cowdria ruminantium* infection in the mouse : a review. *Onderstepoort J. vet. Res.* 54, 267-269.

92. MacKenzie, P.K.I., Van Rooyen, R.E., 1981. The isolation and culture of *Cowdria ruminantium* in albino mice. In Proc. International Conference on « Tick biology and Control », Grahamstown, South Africa, 33-39.

93. Mahan, S.M., Waghela, S.D., McGuire, T.C., Rurangirwa, F.R., Wassink, L.A., Barbet, A.F., 1992. A cloned DNA probe for *Cowdria ruminantium* hybridizes with eight heartwater strains and detects infected sheep. *J. Clin. Microbiol.* 30, 981-986.

94. Mahan, S. M., Tebele, N., Mukwedeya, D., Semu, S., Nyathi, C. B., Wassink, L. A., Kelly, P. J., Peter, T., Barbet, A. F., 1993. An immunoblotting diagnostic assay for heartwater based on the immunodominant 32-kilodalton protein of *Cowdria ruminantium* detects false positives in field sera. *J. Clin. Microbiol.* 31, 2729-2737.

95. Mahan, S.M., Andrew, H.R., Tebele, N., Burridge, M.J., Barbet, A.F. 1995. Immunisation of sheep against heartwater with inactivated *Cowdria ruminantium*. *Res. Vet. Sci.* 58, 46-49.

96. Mahan, S.M., Smith, G.E., Byrom, B., 1994. Concanavalin A-stimulated bovine T-cell supernatants inhibit growth of *Cowdria ruminantium* in bovine endothelial cells *in vitro*. *Infect. Immun.* 62, 747-750.

97. Mahan, S.M., Sileghem, G.E., Smith, G.E., Byrom, B., 1996. Neutralisation of bovine concanavalin A T cell supernatant-mediated anti-*Cowdria* activity with antibodies specific to interferon gamma but not to tumor necrosis factor. *Parasite Immunol.* 18, 317-324.

98. Martinez, D., Swinkels, J., Camus, E., Jongejan, F. 1990. Comparaison de trois antigènes pour le diagnostic de la cowdriose par immunofluorescence indirecte. *Rev. Elev. Méd. vét. Pays trop.* 43, 159-166.

99. Martinez, D., Sheikboudou, C., Couraud, P.O., Bensaïd, A.., 1993a. *In vitro* infection of bovine brain endothelial cells by *Cowdria ruminantium*. *Res. Vet. Sci.* 55, 258-260.

100. Martinez, D., Coisne, S., Sheikboudou, C., Jongejan, F., 1993b. Detection of antibodies to *Cowdria ruminantium* in the serum of domestic ruminants by indirect ELISA. *Rev. Élev, Méd. vét. Trop.* 46, 115-120.

101. Martinez, D., Maillard, J.C., Coisne, S., Sheikboudou, C., Bensaid, A., 1994. Protection of goats against heartwater acquired by immunisation with inactivated elementary bodies of *Cowdria ruminantium*. *Vet. Immunol. Immunopathol.* 41, 153-163.

102. Martinez, D., Perez, J.M., Sheikboudou, C., Debus, A., Bensaid, A., 1996. Comparative efficacy of Freund's and Montanide ISA50 adjuvants for the immunisation of goats against heartwater with inactivated *Cowdria ruminantium*. *Vet. Parasitol.* 67, 175-184.

103. Matheron, G., Barré, N., Camus, E., Gogué, J., 1987. Genetic resistance of Guadeloupe native goats to heartwater. *Onderstepoort J. vet. Res.* 54, 337-340.

104. Matheron, G., Camus, E., Barré, N., Gogué, J., 1991. Résistance à la cowdriose de chèvres créoles en Guadeloupe. Bilan en 1988. *Rev. Élev. Méd. vét. Trop.* N° spécial, 145-153.

105. Mondry, R., Martinez, D., Camus, E., Liebisch, A., Van Vliet, A., Jongejan, F., 1997. Validation of the recombinant MAP1B based ELISA for the detection of antibodies to *Cowdria ruminantium* infection of livestock in the Caribbean. (Submitted).

106. Neitz, W.O., 1939. The immunity in heartwater. *Onderstepoort J. Vet. Sci. Ani. Ind.* 13, 245-283.

107. Neitz, W.O., 1940. Uleron in the treatment of heartwater. *Jl. S. Afr. Vet. Med. Assoc.* 11, 15.

108. Neitz, W.O., 1968. - Heartwater. *Bull. Off. Int. Epiz.* 70, 329-336.

109. Neitz, W.O., Alexander, R.A., 1941. The immunisation of calves against heartwater. *Jl. S. Afr. Vet. Med. Assoc.* 12, 103-111.

110. Neitz, W.O., Alexander, R.A., 1945. Immunisation of cattle against heartwater and the control of the tick-borne diseases, redwater, gallsickness and heartwater. . *Onderstepoort J. Vet. Sci. Ani. Ind.* 20, 137-152.

111. Neitz, A.W.H., Yunker, C.E., 1996. Amino acid and protein depletion in medium of cell cultures infected with *Cowdria ruminantium*. *Ann. N. Y. Acad. Sci.* 791, 17-24.

112. Neitz, W.O., Alexander, R.A., Adelaar, T.F., 1947. Studies on immunity in heartwater. *Onderstepoort J. Vet. Res.* 21, 243.

113. Norval, R.A.I., Andrew, H.R., Yunker. C.E., 1990. Infection rates with *Cowdria ruminantium* of nymphs and adults of the bont tick *Amblyomma hebraeum* collected in the field in Zimbabwe. *Vet. Parasitol.* 36, 277-282.

114. Oberem, P.T., Bezuidenhout, J.D., 1987. The production of heartwater vaccine, *Onderstepoort J. vet. Res.* 54, 485-488.

115. Olivier, J.A., lötter, A.P., Schwulst, W.G.E., Matthee, O., 1988. Chemoprophylaxis of heartwater by using a subcutaneous pill or paste. *Onderstepoort Vet. Res. Inst. Newsl.* 6, 7-9.

116. Palmer, B.A., Hetrick, F.M., Jerrels, T.R., 1984. Gamma interferon production in response to homologous and heterologous strain antigens in mice chronically infected with *Rickettsia tsutsugamushi*. *Infect. Immun.* 46, 237-244.

117. Park, J., Rikihisa, Y., 1992. L-arginine-dependent killing of intracellular *Ehrlichia risticii* by macrophages treated with gamma interferon. *Infect. Immun.* 60, 3504-3508.

118. Perreau, P., Morel, P.C., Barré, N., Durand, P., 1980. Existence de la cowdriose (Heartwater) à *Cowdria ruminantium,* chez les petits ruminants des Antilles françaises (la Guadeloupe) et des Mascareignes (la Réunion et l'Ile Maurice). *Rev. Elev. Méd. vét. Pays Trop.* 33, 21-22.

119. Peter, T.F., S.L. Deem, A.F. Barbet, R.A.I. Norval, B.H. Simbi, P.J. Kelly, and S.M. Mahan. 1995. Development and evaluation of PCR assay for detection of low levels of *Cowdria ruminantium* infection in *Amblyomma* ticks not detected by DNA probe. *J. Clin. Microbiol.* 33, 166-172

120. Pienaar, J.G., 1970. Electron microscopy of *Cowdria* (*Rickettsia*) *ruminantium* (Cowdry, 1926) in the endothelial cells of the vertebrate host. *Onderstepoort J. Vet. Res.* 37, 1, 67-68.

121. Poole, J.D.H., 1962a. Flock immunisation of sheep and goats against heartwater. Part I : investigations regarding routine flock immunisation of sheep. *Jl. S. Afr. Vet. Med. Assoc.* 33, 35-41.

122. Poole, J.D.H. 1962b. Flock immunisation of sheep and goats against heartwater. Part II : preliminary experiments on flock immunisation of goats. *Jl. S. Afr. Vet. Med. Assoc.* 33, 357-362.

123. Provost, A., Bezuidenhout, J.D., 1987. The historical background and global importance of heartwater. *Onderstepoort J. vet. Res.* 54, 165-169.

124. Prozesky, L., 1987a. Heartwater. The morphology of *Cowdria ruminantium* and its staining characteristics in the vertebrate host and *in vitro*. *Onderstepoort J. vet. Res.* 54, 173-176.

125. Prozesky, L., 1987b. The pathology of heartwater. III. A review. *Onderstepoort J. vet. Res.* 54, 281-286.

126. Prozesky, L., Du Plessis, J.L., 1985. The pathology of heartwater II. A study of the lung lesions in sheep and goats infected with the Ball 3 strain of *Cowdria ruminantium*. *Onderstepoort J. vet. Res.* 52, 81-85.

127. Prozesky, L., Bezuidenhout, J.D., Paterson, C.L., 1986. Heartwater : an *in vitro* study of the ultrastructure of *Cowdria ruminantium*. *Onderstepoort J. vet. Res.* 53, 153-159.

128. Purchase, H.S. 1945. A simple and rapid method for demonstrating *Rickettsia ruminantium* (Cowdry, 1925), in heartwater brains. *Vet. Rec.* 57, 413-414.

129. Reddy, G.R., Sulsona, C.R., Harrison, R.H., Mahan, S.M., Burridge, M.J., Barbet, A.F., 1996. Sequence heterogeneity of the major antigenic protein 1 genes from *Cowdria ruminantium* isolates from different geographical areas. *Clin. Diagn. Lab. Immunol.* 3, 417-422.

130. Sahu, S.P., 1986. Fluorescent antibody technique to detect *Cowdria ruminantium* in *in-vitro*-cultured macrophages and buffy coats from cattle, sheep and goats. *Am. J. Vet. Res.* 47, 6 1253-1257.

131. Sahu, S.P., Dardiri, A.H., Wool, S.H., 1983. Observation of *Rickettsia ruminantium* in leukocytic cell cultures from heartwater-infected goats, sheep and cattle. *Am. J. vet. Res.* 44, 1093-1097.

132. Scott, G.R. 1987. The taxonomic status of the causative agent of heartwater. *Onderstepoort J. Vet. Res.* 54, 257-260.

133. Shemer, Y., Sarov, I., 1985. Inhibition of growth of *Chlamydia trachomatis* by human gamma interferon. *Infect. Immun.* 48, 592-596.

134. Soldan, A.W., T.L. Norman, S. Masaka, E.A. Paxton, R.M. Edelstein and K. Sumption. 1993. Seroconversion to *Cowdria ruminantium* of Malawi zebu calves, reared under different tick control strategies. . *Rev. Élev. Méd. vét. Trop.* 46, 171-177.

135. Spreull, J., 1904. Heartwater inoculation experiments. *Agric. J. Cape of Good Hope*, 24, 433-442.

136. Spreull, J., 1922. Heartwater. *J. Dep. Agri., Union of South Africa*, 4, 236-245.

137. Stewart, C.G., 1987a. Specific immunity in farm animals to heartwater. *Onderstepoort J. vet. Res.* 54, 341-342.

138. Stewart, C.G., 1987b. Specific immunity in mice to heartwater. *Onderstepoort J. vet. Res.* 54, 343-344.

139. Synge, B.A. 1978. Brain biopsy for the diagnosis of heartwater. *Trop. Anim. Hlth Prod.* 10, 45-48.

140. Theiler, A., 1906. Immunisation against heartwater. In Annual report of the Director of Agriculture (1904-1905), Transvaal, South Africa, 121-122.

141. Thomas, A.D., Mansvelt, P.R., 1957. Immunisation of goats against heartwater. *Jl S. Afr. Vet. Med. Assoc.* 28, 163-168.

142. Totté, P., De Gee, A.L.W., Wérenne, J., 1989. Possible role of interferon in the resistance of cattle against the rickettsiale *Cowdria ruminantium* during vaccination. *J. Cell. Biochem.* Supp., 13E, 112.

143. Totté, P., Blankaert, D., Zilimwabagabo, P., Wérenne, J., 1993. Inhibition of *Cowdria ruminantium* yield by interferons alpha and gamma in endothelial cells. *Rev. Elev. Méd. Vét. Pays trop.* 46, 189-194.

144. Totté, P., Jongejan, F., De Gee, A.L.W., Wérenne, J., 1994a. Production of alpha interferon in *Cowdria ruminantium*-infected cattle and its effect on infected endothelial cell cultures. *Infect. Immun.* 62, 2600-2604.

145. Totté, P., Moguilevsky, N., Vachiéry, N., Tournay, C., Bollen, A., Wérenne, J., 1994b. Protective *in vitro* effect against murine rickettsia infection by recombinant myeloperoxidase produced in CHO cells. In : KOBAYASHI, T., KITAGAWA, Y., OKUMURA, K. Animal Cell Technology : basic and applied aspects, vol. 6, 549-553. Kluwer Academic Publ.

146. Totté, P., Vachiéry, N., Heremans, H., Billiau, A., Wérenne, J., 1994c. Protection against *Cowdria ruminantium* infection in mice with gamma interferon produced in animal cells. In : KOBAYASHI, T., KITAGAWA, Y., OKUMURA, K. Animal Cell Technology : basic and applied aspects, vol. 6, 595-599. Kluwer Academic Publ.

147. Totté, P., Vachiéry, N., Martinez, D., Trap, I., Ballingdall, K.T., MacHugh, N.D., Bensaid, A., Wérenne, J., 1996. Recombinant bovine interferon gamma inhibits the growth of *Cowdria ruminantium* but fails to induce major histocompatibility complex class II following infection of endothelial cells. *Vet. Immunol. Immunopathol.* 53, 61-71.

148. Totté, P., MacKeever, D., Martinez, D., Bensaid, A., 1997. Protection of cattle against cowdriosis by vaccination with inactivated elementary bodies of *Cowdria ruminantium*. *Infect. Immun.* 65, 236-241.

149. Turco, J., Winkler, H., 1984. Effect of mouse lymphokines and cloned mouse interferon-γ on the interaction of *Rickettsia prowazekii* with mouse macrophage-like RAW264.7 cells. *Infect. Immun.* 45, 303-308.

150. Turco, J., Winkler, H., 1990. Interferon α/β and *Rickettsia prowazekii* : induction and sensitivity. *Annals N. Y. Aca. Sci.* 590, 168-186.

151. Uilenberg, G. 1971a. Etudes sur la cowdriose à Madagascar: première partie. *Rev. Elev. Méd. vét. Pays trop.* 24, 355-364.

152. Uilenberg, G. 1982. Experimental transmission of *Cowdria ruminantium* by the Gulf Coast tick *Amblyomma maculatum*: Danger of introducing heartwater and benign African theileriasis onto the American mainland. *Am. J. Vet. Res.* 43, 1279-1282.

153. Uilenberg, G., 1983. Heartwater (*Cowdria ruminantium* infection) : current status. *Adv. vet. Sci. Comp. Med.* 27, 427-480.

154. Uilenberg, G., 1995. International collaborative research : significance of tick-borne hemoparasitic diseases to world animal health. *Vet. Parasitol.* 57, 19-41.

155. Uilenberg, G., 1996. Progress and priorities in research on heartwater. *Ann. N. Y. Aca. Sci.* 791, 1-16.

156. Uilenberg, G., Camus, E., 1993. Heartwater (cowdriosis). In : Woldehiwet, Z., Miodrag, R. Rickettsial and Chlamydial diseases of domestic ruminants. Pergamon Press. p 293-332.

157. Uilenberg, G., Zivkovic, D., Dwinger, R.H., Ter Huurne, A.H.M., Perie, N.M., 1983. Cross immunity between strains of *Cowdria ruminantium*. *Res. Vet. Sci.* 35, 200-205.

158. Uilenberg, G., Barré, N., Camus, E., Burridge, M.J., Garris, G.I., 1984. Heartwater in the Caribbean. 4th Internat. Conf. of Institutions of tropical Veterinary Medicine. *Prev. vet. Med.* 2, 255-267.

159. Uilenberg, G., Camus, E., Barre, N., 1985. Quelques observations sur une souche de *Cowdria ruminantium* isolée en Guadeloupe (Antilles Françaises). *Rev. Elev. Méd. Vét. Pays trop.* 38, 34-42.

160. Wassink, L.A., Franssen, F.F.J., Uilenberg, G., Périé, N.M., 1987. Observations on mouse-infective stocks of *Cowdria ruminantium* : attempts to demonstrate persistence of the organism in mice immune to the Kwanyanga stock. *Res.Vet. Sci.* 42, 1, 65-67.

161. Van de Pypekamp, H. and L. Prozesky. 1987. Heartwater. An overview of the clinical signs, susceptibility and differential diagnoses of the disease in domestic ruminants. *Onderstepoort J. Vet. Res.* 54, 263-266.

162. Van Der Merwe, L., 1979. Field experience with heartwater *(Cowdria ruminantium)* in cattle. *Jl S. Afr. Vet. Assoc.* 50, 323-325.

163. Van Der Merwe, L., 1987. The infection and treatment method of vaccination against heartwater. *Onderstepoort J. Vet. Res.* 54, 489-491.

164. Van Kleef, M., Neitz, A.W.H., De Waal, D.T., 1993. Isolation and characterization of antigenic proteins of *Cowdria ruminantium*. *Rev. Elev. Méd. Vét. Pays trop.* 46, 157-164.

165. Van Winkelhoff, A.J., Uilenberg, G., 1981. Heartwater : cross-immunity studies with strains of *Cowdria ruminantium* isolated in West and South Africa. *Trop. Anim. Hlth Prod.* 13, 160-164.

166. Van Vliet, A. H. M., F. Jongejan, and B. A. M. van der Zeijst. 1992. Phylogenetic position of *Cowdria ruminantium* (*Rickettsiales*) determined by analysis of amplified 16S ribosomal DNA seqences. *Int. J. Syst. Bact.* 42, 494-498.

167. Van Vliet, A. H. M., F. Jongejan, M. van Kleef and B. A. M. van der Zeijst. 1994. Molecular cloning, sequence analysis and expression of the gene encoding the Major Antigenic Protein of *Cowdria ruminantium*. *Infect. Immun.* 62, 1451-1456.

168. Van Vliet, A. H. M., B. A. M. van der Zeijst, E. Camus, S. M. Mahan, D. Martinez, and F. Jongejan. 1995. Use of a specific immunogenic region on the *Cowdria ruminantium* MAP1 protein in a serological assay. *J. Clin. Microbiol.* 33, 2405-2410.

169. Walker, J.B. Olwage, A., 1987. The tick vectors of *Cowdria ruminantium* (*Ixodoidae*, *Ixodidae*, genus *Amblyomma*) and their distribution. *Onderstepoort J. Vet. Res.* 54, 353-379.

170. Waghela, S.D., Rurangirwa, F.R., Mahan, S.M., Yunker, C.E., Crawford, T.B., Barbet, A.F., Burridge, M.J., McGuire, T.C., 1991. A cloned DNA probe identifies *Cowdria ruminantium* in *Amblyomma variegatum* ticks. *J. Clin. Microbiol.* 29, 2571-2577.

171. Wassink, L.A., Franssen, F.F.J., Uilenberg, G., Périé, N.M., 1987. Observations on mouse-infective stocks of *Cowdria ruminantium* : attempts to demonstrate persistence of the organism in mice immune to the Kwanyanga stock. *Res.Vet. Sci.* 42, 1, 65-67.

172. Wassink, L.A., Jongejan, F., Gruys, E., Uilenberg, G., 1990. Observations on mouse-infective stocks of *Cowdria ruminantium* : microscopical demonstration of the Kwanyanga stock in mouse tissue and the carrier status of the Senegal stock in mice. *Res. vet. Sci.* 48, 389-390.

173. Yunker, C.E., Byrom, B., Semu, S., 1988. Cultivation of *Cowdria ruminantium* in bovine vascular endothelial cells. *Kenya vet.* 12, 12-16.

174. Yunker, C.E., Mahan, S.M., Waghela, S.D., McGuire, T.C., Rurangirwa, F.R., Barbet, A.F., Wassink, L.A., 1993. Detection of *Cowdria ruminantium* by means of a DNA probe, pCS20 in infected bont ticks, *Amblyomma hebraeum*, the major vector of heartwater in Southern Africa. *Epidemiol. Infect.* 110, 95-104.

175. Yunker, C.E., 1996. Heartwater in sheep and goats : a review. *Onderstepoort J. vet. Res.* 63, 159-170.

AIM AND OUTLINE OF THE THESIS

Studies on the nature of immunity in heartwater, the development of efficient vaccines and reliable diagnostic tests, have long been hampered by the lack of a suitable antigen due to failure to cultivate the causal agent *in vitro* until 1985.

The aim of this thesis was to try to understand immune mechanisms leading to protection or pathology in heartwater, and to develop new methods to vaccinate domestic ruminants against the disease. Methods to evaluate the extent of diversity among *Cowdria* isolates were also developed.

Cowdria ruminantium infection in susceptible ruminants is characterized in most cases by central nervous signs and a non absolute tropism of the pathogen for endothelial cells of brain capillaries. This led to the development of the first diagnostic method consisting of brain squash smear staining. In **Chapter 2**, we describe our studies on the ability of *Cowdria* to infect and grow *in vitro* in bovine brain endothelial cells (BBEC) in order to be able to conduct investigations on immune and pathological mechanisms in comparison with other types of endothelial cells.

Using these BBEC, a study was made on the synthesis of the pro-inflammatory cytokines IL-1, IL-6, IL-8 and on BoLA class I and II mRNA, after infection by *C. ruminantium* as described in **chapter 4**. Infection by *Cowdria* induced the synthesis of IL-1, IL-6 and IL-8 mRNA. However, infection by *Cowdria* did not affect the synthesis of Major Histocompatibility Complex (MHC) class I, class II DRα, DRβ, DQβ and invariant chain mRNA, but induced the synthesis of an unusual DQα transcript.

Cowdria ruminantium is an obligate intracellular bacterium. For this reason, it has long been thought that the protective immunity in heartwater is mainly based on cytotoxicity by T cells. This type of immunity is based on the presentation of parasite antigens to cytotoxic T lymphocytes in an MHC class I restricted manner. This would imply that parasites live and multiply within the infected host cell. In **chapter 3**, we demonstrate for the first time the possibility to immunise susceptible goats by inoculation of killed parasites emulsified in Freund's adjuvant. This introduce a new concept of vaccine development for cowdriosis based on inactivated antigens.

Freund's adjuvant is a very potent experimental adjuvant with considerable side-effects that make it inappropriate for inclusion in a vaccine for use in the field. In **chapter 6** we have compared the efficacy of Montanide ISA50, a licenced oil

adjuvant, and Freund's adjuvant for their ability to confer protection to susceptible goats when incorporated in an inactivated vaccine. Both adjuvants were equivalent. These results are a promising first step toward a first generation inactivated vaccine.

The differentiation of the phenotype of the subpopulations of circulating immunocompetent cells during the whole process of immunisation and challenge of goats described in chapter 6, was followed by flow cytometry. The results reported in **chapter 8**, show a spectacular raise of monocytes and $CD8^+$ T cells after challenge. This raises the question of the function of these $CD8^+$ T cells and also the role of the monocytes which are strongly activated during the peak of blood mononuclear cells after virulent challenge.

The experimental inactivated vaccine containing Freund's adjuvant initially developed for the immunisation of goats was tested successfully in cattle. The results are reported in **chapter 7** together with the analysis of the immune responses developed by the animals. In addition to a phenotypic analysis of the circulating mononuclear cells similar to that conducted in goats, functionnal analysis showed a specific activation of $CD4^+$ cells by *Cowdria* antigens with secretion of interferon-γ. *Cowdria* specific $CD4^+$ T-cell lines were derived for later *in vitro* screening of candidate vaccine antigens.

Protective immune responses against intracellular parasites are often Th1-type responses in which interferon-γ is of major importance. The role of this cytokine in the inhibition of the growth of many *Rickettsia* species *in vitro* and in the protective response *in vivo*, has been extensively described. The analysis conducted in **chapter 5** showed that interferon-γ was a potent inhibitor of *Cowdria* growth in endothelial cells.

Parasites have also developed strategies to evade immune responses in particular by down-regulating the expression at the cell surface of MHC molecules presenting antigens to lymphocytes. The study reported in chapter 4 at the RNA level was in part completed in **chapter 5** by experiments showing a down regulation of the expression of MHC class II molecules at the surface of endothelial cells infected by *Cowdria*.

As important as the understanding of immune mechanisms for the development of vaccines, is the evaluation of the antigenic variability of the pathogens. So far the only way to type *Cowdria* isolates were cross-protection tests. Although very informative, these tests are becoming unworkable with the increasing number of isolates to test. In **chapter 9** the application of a RAPD method to evaluate the extent of the genetic diversity of *Cowdria*, is described. Although the genetic diversity does not necessarily correlates with antigenic diversity, this typing method

makes possible to cluster strains to be tested in cross-protection. Sequencing of RAPD fragments can also be used for genetic analysis and to develop molecular tools for epidemiology.

All studies on cowdriosis have so far been conducted on uncloned isolates. Cloned parasites would give much better defined results in immunological studies. The development of a method for cloning *Cowdria* and its validation by RAPD is reported in **chapter 10**.

CHAPTER 2

In vitro Infection of bovine brain endothelial cells by *Cowdria ruminantium*

D. Martinez[a], C. Sheikboudou[a], P.O. Couraud[b], A. Bensaid[c].

[a] *Institut d'Elevage et de Médecine Vétérinaire des Pays Tropicaux (IEMVT-CIRAD), BP 1232, 97184 Pointe à Pitre Cedex, Guadeloupe, West Indies.*

[b] *Laboratoire d'Immuno-pharmacologie Moléculaire, Institut Cochin de Génétique Moléculaire (ICGM), 22 Rue Méchain, 75014 Paris, France.*

[c] *Laboratoire d'Immunologie, Institut d'Elevage et de Médecine Vétérinaire des Pays Tropicaux, 10 Rue Pierre Curie, 94704 Maisons-Alfort Cedex, France.*

Research in Veterinary Science, 1993, 55, 258-260.

Abstract

Endothelial cells from bovine brain capillaries (BBEC) were successfully infected with the Gardel stock of *Cowdria ruminantium*. Growth conditions of *Cowdria* in BBEC and bovine umbilical endothelial cells (BUE) were identical with average intervals between passages of 8.6 and 8.9 days in BBEC (17 passages) and BUE (28 passages) respectively. The time required to complete the life cycle and the morphology of the parasite were identical in both types of cells. The demonstration that BBEC may be infected with *Cowdria ruminantium* offers the means to study the mechanism of infection of the blood-brain barrier endothelium by this parasite.

Cowdria ruminantium is the causative agent of cowdriosis, an important disease of wild and domestic ruminants which is transmitted by several species of *Amblyomma* ticks (Uilenberg, 1983). The disease is widespread in subsaharan Africa (Provost and Bezuidenhout, 1987) and was identified more recently in 3 Caribbean islands (Perreau et al., 1980; Birnie et al., 1984). It is characterized by high fever and central nervous signs leading to death within a few hours or days in susceptible animals. *C. ruminantium* is an obligatory parasite of endothelial cells although its presence in neutrophils was demonstrated by Logan et al. (1987) during the febrile phase of the disease. The demonstration of rickettsiae in capillary endothelial cells of brain smears of infected animals (Purchase, 1945) is the most widely used method of diagnosis. However, except for some particular stocks (Sénégal and Welgevonden stocks), colonies of parasites are very rare in capillaries and there is no correlation between the number of colonies and the severity of nervous signs. The disease lacks safe vaccines and its pathogenicity remains widely unexplained basically because of unavailability of *in vitro* cultivation methods until recently. In 1985, the cultivation of *C. ruminantium* in bovine umbilical endothelial cells (BUE) was described (Bezuidenhout et al., 1985). Since that time, *Cowdria* has commonly been grown in BUE, bovine aorta and pulmonary artery endothelial cells (Yunker et al., 1988; Byrom and Yunker, 1990; Jongejan et al., 1990; Martinez et al., 1990; Byrom et al., 1991). However, endothelial cells have various properties depending on their localisation in the body (large vessels vs capillaries, peripheral vs central vessels). Therefore, an appropriate *in vitro* model is needed to study the nervous pathology of cowdriosis. The purpose of this study was to demonstrate the possibility of infecting bovine brain capillary endothelial cells (BBEC) with *C. ruminantium* in order to develop an *in vitro* system to study the pathological action of this parasite on bovine brain.

Endothelial cells were isolated from bovine brain as previously described (Gospodarovicz et al., 1986). They were grown on 0.2 per cent (w/v) gelatin-coated flasks in Dulbecco's modified minimum essential medium (DMEM) (Seromed) supplemented with 2 mM glutamine, 10 per cent fetal calf serum (FCS) (Seromed), 100 iu ml^{-1} penicillin G, 100 µg ml^{-1} streptomycin, 1 ng ml^{-1} basic fibroblast growth factor (bFGF) (Boehringer Mannheim). For cell infection, the medium was either DMEM supplemented with 2.95 mg ml^{-1} tryptose phosphate broth (TPB) (Difco), or Glasgow modified Eagle's minimum essential medium (GMEM) (Seromed) supplemented with glutamine, penicillin G, streptomycin, FCS and TPB at the above mentioned concentrations (Bezuidenhout et al., 1985). Attempts were also made to cultivate *Cowdria* in GMEM without TPB. GMEM was not supplemented with bFGF.

The Gardel stock of *C. ruminantium* isolated in Guadeloupe (Uilenberg et al., 1985) was used for infections of BBEC. BBEC were inoculated with the supernatant of the 25th passage of *C. ruminantium* in BUE cells. The BUE cells were originally

infected with the blood of an experimental infected goat (Martinez et al., 1990). When a strong cytopathogenic effect (CPE) appeared (approximately 80-90% of cell lysis), supernatants of infected cultures were collected and used to inoculate confluent monolayers of BBEC. After addition of the inoculum (10 ml per 75cm^2 flask), the flasks were incubated at 37°C for two hours on a rocking platform. Fresh medium was then added and the cultures were monitored daily for the appearance of CPE. Passages between infected and non infected BBEC cultures were performed in a similar manner. Passages of *Cowdria* in BUE cells were carried out concurrently with BBEC to compare the culture conditions in both types of cells. The number of days between the inoculation of cells and the first CPE detected, and the time between the inoculation and the next passage were recorded for each culture.

The presence of *C. ruminantium* in cultures was confirmed by staining with eosin-methylene blue (RAL 555) and an indirect fluorescent antibody test (IFAT). The IFAT was performed as follows. When a strong CPE appeared, the bottom of flasks with remaining adherent cells was cut into slides and the cells fixed in 80 per cent acetone for 10 minutes at -20°C. After drying under a laminar flow hood, slides were covered with a positive goat serum diluted 1/100 in phosphate buffer saline (PBS) and incubated 30 minutes at 37°C in a humid chamber. Slides were washed three times (five min each) in PBS and covered with FITC conjugate rabbit antigoat IgG immunoglobulin diluted 1/100 in PBS containing 0.2 per cent Evans blue as a counterstain. Slides were washed again (three times for five minutes in PBS) and mounted with Fluoprep (Bio-Mérieux) for examination with an epifluorescence microscope at a X 400 magnification.

BBEC were successfully infected with the Gardel strain of *C. ruminantium*. One passage was performed in DMEM and 17 in GMEM. In all cases, CPE in BBEC monolayers appeared in 4 to 7 days (mean 5 days, number of observations n = 15), and *Cowdria* were passaged between 6 and 14 days (mean 8.6 days, n = 17) after the infection of cells. These values were not significantly different when *Cowdria* was cultivated in BUE : time to the first detection of CPE was 4 to 7 days (mean 4.6, n = 22) and time between passages was 6 to 13 days (mean 8.9, n = 28). The organism was also successfully passaged 6 times without TPB. The CPE consisted of small plaques of cell lysis which became confluent within a few days. Morulae and elementary bodies of *Cowdria* were observed in all cultures after staining with RAL 555 and their specificity confirmed by IFAT.

Endothelial cells were infected without the need to retard cell growth (γ-irradiation, cycloheximide) or to facilitate contact between cells and parasites (centrifugation, polybrene) as described in the original technique (Bezuidenhout et al., 1985; Bezuidenhout, 1987). This is in agreement with results obtained in other types of endothelial cells (Yunker et al., 1988; Byrom and Yunker, 1990; Jongejan et al., 1990; Martinez et al., 1990; Byrom et al., 1991) which show that methods commonly used to infect cells with chlamydiae or rickettsiae (Weiss and Dressler,

1960; Benes and McCormack, 1982) are not necessary for *Cowdria*. This is the second report of the successful cultivation of *Cowdria* in DMEM (Byrom and Junker, 1990). However, in both cases the medium was supplemented with TPB which was considered to have a protecting effect on the parasite. The failure of *Cowdria* cultivation until 1985 could be attributed to the absence of such a protecting agent in the culture medium. Previously, the isolation of *C. ruminantium* in several primary endothelial cell from bovine umbilical cord, bovine and goat aorta, cultivated in RPMI without TPB was unsuccessful using the same method, with or without γ-irradiation at 45 or 90 GY (D. Martinez, unpublished observations). However, in this study, the parasite was successfully passaged six times without TPB and there were no differences between these cultures and cultures with TPB. These results suggest that TPB acts as a protecting agent for *Cowdria* when isolating this parasite from affected animals, but that it becomes unnecessary after several passages in culture probably due to selection of well-growing *Cowdria*. Byrom and Yunker (1990) found that Leibovitz's L-15 medium supplemented with 0.45 per cent glucose was better than GMEM for isolation of *Cowdria* and that the average time between passages was reduced from 12.1 to 8.8 days with these 2 media respectively. In the present conditions, GMEM allowed passages every 8.6 and 8.9 days in BBEC and BUE cells, respectively. The morphology and the cycle of *Cowdria* were identical in these 2 types of cells.

In conclusion, the *in vitro* infection of endothelial cells from with *C. ruminantium* offers the means to study the pathogenicity of cowdriosis and the mechanisms of infection of the cerebral capillary endothelium.

Acknowledgements

Part of this research was supported by the European Community under contract TS2-115-C.

References

1. Benes, S., McCormack, W.M., 1982. Comparison of methods for cultivation and isolation of *Chlamydia trachomatis*. *J. Clin. Microbiol.* 16, 847-850.

2. Bezuidenhout, J.D., Paterson, C.L., Barnard, B.J.D., 1985. *In vitro* cultivation of *Cowdria ruminantium*. *Onderstepoort J. Vet. Res.* 52, 113-120.

3. Bezuidenhout, J.D., 1987. The present state of *Cowdria ruminantium* cultivation in cell lines. *Onderstepoort J. Vet. Res.* 54, 205-210.

4. Birnie, E.L., Burridge, M.J., Camus, E., Barré, N., 1984. Heartwater in the Caribbean : isolation of *Cowdria ruminantium* from Antigua. *Vet. Record* 116, 121-123.

5. Byrom, B., Yunker, C.E., 1990. Improved culture conditions for *Cowdria ruminantium* (Rickettsiales), the agent of heartwater disease of domestic ruminants. *Cytotechnology* 4, 285-290.

6. Byrom, B., Yunker, C.E., Donovan, P.L., Smith, G.E., 1991. *In vitro* isolation of *Cowdria ruminantium* from plasma of infected ruminants. *Vet. Microbiol.* 26, 263-268.

7. Gospodarovicz, D., Massoglia, S., Cheng, J., Fujii, D.K., 1986. Effects of fibroblast growth factor and lipoproteins on the proliferation of endothelial cells derived from bovine adrenal cortex, brain cortex and corpus luteum capillaries. *J. Cell. Physiol.* 127, 121-136.

8. Jongejan, F., Thielemans, M.J.C., 1990. Antigenic analysis of the tick-borne rickettsia *Cowdria ruminantium*. In : *Proceedings of the 6th international conference of institutes for tropical veterinary medicine, Wageningen, The Netherlands, 28 august-1 september 1989*, p 305-309.

9. Logan, L.L., Whyard, T.C., Quintero, J.C., Mebus, C.A., 1987. The development of *Cowdria ruminantium* in neutrophils. *Onderstepoort J. Vet. Res.* 54, 197-204.

10. Martinez, D., Swinkels, J., Camus, E., Jongejan, F., 1990. Comparaison de trois antigènes pour le diagnostic de la cowdriose par immunofluorescence indirecte. *Rev. Elev. Méd. Vét. Pays Trop.* 43, 159-166.

11. Perreau, P., Morel, P.C., Barré, N., Durand, P., 1980. Existence de la cowdriose (heartwater) à *Cowdria ruminantium* chez les ruminants des Antilles françaises (La Guadeloupe) et des Mascareignes (La Réunion et île Maurice). *Rev. Elev. Méd. Vét. Pays Trop.* 33, 21-22.

12. Provost, A., Bezuidenhout, J.D., 1987 The historical background and global importance of heartwater. *Onderstepoort J. Vet. Res.* 54, 165-169.

13. Purchase, H.S., 1945. A simple and rapid method for demonstrating *Rickettsia ruminantium* (Cowdry, 1925), in heartwater brains. *Vet. Record* 57, 413-414.

14. Uilenberg, G., 1983. Heartwater (*Cowdria ruminantium* infection) : Current status. *Adv. Vet. Sci. Comp. Med.* 27, 427-480.

15. Uilenberg, G., Camus, E., Barré, N., 1985. Quelques observations sur une souche de *Cowdria ruminantium* isolée en Guadeloupe (Antilles françaises). *Rev. Elev. Méd. Vét. Pays Trop.* 38, 34-42.

16. Yunker, C.E., Byrom, B., Semu, S., 1988. Cultivation of *Cowdria ruminantium* in bovine vascular endothelial cells. *Kenya Vet.* 12, 12-16.

17. Weiss, E., Dressler, H.R., 1960. Centrifugation of rickettsiae and viruses onto cells and its effect on infection. *Proceedings of the Society for Experimental Biology and Medicine* 103, 691-695.

CHAPTER 3

Protection of goats against heartwater acquired by immunisation with inactivated elementary bodies of *Cowdria ruminantium*

D. Martinez[a], J.C. Maillard[a], S. Coisne[a], C. Sheikboudou[a], A. Bensaid[b].

[a] *Centre International en Recherche Agronomique pour le Développement, Département d'Elevage et de Médecine Vétérinaire des Pays Tropicaux (CIRAD-EMVT), BP 1232, 97184 Pointe à Pitre Cedex, Guadeloupe, West Indies.*

[b] *CIRAD-EMVT, 10 rue Pierre Curie, 94704 Maisons-Alfort Cedex, France.*

Veterinary Immunology and Immunopathology, 1994, 41, 153-163.

Abstract

In two experiments, four and five goats were vaccinated by giving two subcutaneous injections of a preparation of inactivated elementary bodies of *Cowdria ruminantium* (Gardel stock) mixed with Freund's adjuvant. All vaccinated animals together with four naive controls were challenged intravenously with 5 ml of supernatant of a culture of bovine endothelial cells infected with the same stock of *Cowdria*. All goats developed a high temperature. Two out of four, and four out of five vaccinated goats survived the challenge whereas all naive control animals died within 7 to 12 days. Vaccinated goats which died, survived longer than the controls. No difference in antibody titres was observed between protected and non-protected vaccinated goats. Moreover, immune sera from surviving goats, whether heat inactivated or not, were unable to neutralize the infection of bovine endothelial cells by *Cowdria in vitro*. Mechanisms conferring protection on the immunized goats are unknown at the moment but the hypothesis that T-helper lymphocyte populations have been elicited seems to be likely. This method of immunization with dead organisms will help in the search of protective antigens against cowdriosis.

Introduction

Cowdriosis commonly known as heartwater is a disease affecting wild and domestic ruminants distributed in the subsaharan regions of Africa (Provost and Bezuidenhout, 1987) and in several islands of the Indian Ocean (Uilenberg, 1983; Du Plessis et al., 1989; Flach et al., 1990) and in the lesser Antilles (Perreau et al., 1980; Birnie et al., 1984; Uilenberg et al., 1984), from where it threatens the American mainland (Barré et al. 1987). The causative organism, *Cowdria ruminantium* (Rickettsiales) is transmitted by ticks belonging to the genus *Amblyomma* (Uilenberg, 1983). It is an obligate parasite of endothelial cells, especially of brain capillaries, although its presence in circulating neutrophils was demonstrated by Logan et al. (1987). In susceptible animals, the disease is characterized by high hyperthermia and central nervous symptoms which generally lead to death. Despite the fact that the parasite was described in 1925 by Cowdry, the development of serological tests and vaccines has long been impaired by the inability to cultivate the parasite *in vitro*. In the absence of proper vaccines, protection of animals is achieved by injection of infected blood or tick homogenates followed by treatment of the reacting animals with tetracyclin (Bezuidenhout, 1989). Such vaccinated animals, as well as animals recovering from a natural disease, exhibit a prolonged and solid immunity to cowdriosis. This suggests that the development of vaccines to control the disease may be feasible.

The cultivation of *Cowdria* in bovine endothelial cells was described by Bezuidenhout et al. (1985), and the attenuation of the Senegal stock of the parasite was obtained by serial passages in culture (Jongejan, 1991). However, attempts at attenuating other stocks of *Cowdria* have failed. Thus, although the protection of susceptible ruminants can be improved by using this attenuated stock in certain regions, it cannot be considered as a universal vaccine against heartwater. In effect, the immunity induced by a stock of *C. ruminantium* does not necessarily confer protection against infection with another stock (Jongejan and Thielemans, 1991). This antigenic diversity among *C. ruminantium* isolates must be taken into account in the development of vaccines against heartwater. Moreover, live vaccines should not be used to protect uninfected threatened areas. The identification of relevant antigens appears to be essential for the development of efficient and safe vaccines. In this study, experiments were carried out in order to investigate the ability of inactivated *Cowdria* mixed with adjuvant to induce protection of goats against a challenge with a virulent strain of *C. ruminantium*.

Materials and methods

Experimental animals

Seventeen goats from Les Saintes, Islands from the lesser Antilles free of *Amblyomma* ticks and cowdriosis, were used for vaccination trials. All animals were aged 1.5 to 2 years and weighed between 12 and 15 kg. They were maintained on tick proof duckboards throughout the experiments and screened for antibody using enzyme-linked immunosorbent assay (ELISA) to confirm the absence of previous contact with *C. ruminantium*.

Rickettsial stock

The Gardel stock of *C. ruminantium* isolated in Guadeloupe (Uilenberg et al., 1985) was used for the preparation of vaccine antigens and for virulent infections of goats (blood stabilate and cell culture supernatant).

Antigens

Cowdria ruminantium was produced in bovine umbilical endothelial cells (BUE) as described previously (Martinez et al., 1990). Antigens were prepared from passages 21 and 42 of the parasite in culture in Experiments 1 and 2, respectively. The elementary bodies were obtained from the supernatants of eight flasks (175 cm^2) of infected cultures as described below. When the cell monolayers showed a cytopathogenic effect consisting of 80-90% cell lysis, the supernatants were collected, pooled (total volume 800 ml) and centrifuged at 1300 g for 15 min to spin down the cell debris. The supernatant containing free elementary bodies (EB) was saved. The pellet still containing a large amount of EB was resuspended in 40 ml phosphate buffer saline (PBS) 0.1M, pH 7.2, shaken vigorously and centrifuged at 1300 g for 15 min. This procedure was repeated three times and the supernatants saved each time. Finally, all supernatants were pooled and centrifuged at 16 000 g for 40 min. The pellet containing the EB was suspended in 5 ml PBS and the protein content determined by the method of Bradford (1976). Sodium azide (0.1%) was added to the suspension which was kept overnight at +4°C and then frozen at -20°C until used.

The purity of the antigen preparation was verified by indirect immunofluorescence as described below. Spots of 5 µl of antigen were allowed to dry on slides which were then fixed in cold acetone for 10 min. The antigen was incubated at 37°C for 30 min with a positive goat serum diluted at 1/100 in PBS. A negative control was run on each slide. After washing, the slide was covered with a fluorescein isothiocyanate (FITC) conjugated rabbit anti-goat immunoglobulin (H+L

chains, NORDIK Immunology, Tilburg, Netherlands) diluted 1/100 in PBS and incubated at 37°C for 30 min. The slide was then mounted in fluoprep (Mérieux) and examined with an epifluorescence microscope at 400X magnification.

At the time of immunization, the suspension was thawed and used as antigen either without further treatment or after five cycles of sonication (30 s each) interspersed by intervals of 1 minute in an ethanol-ice bath. These preparations were designated whole elementary body antigen (WEB) and sonicated elementary body antigen (SEB) respectively.

Immunisation procedure

Experiment 1

Two goats (numbers 9124 and 9126) were injected subcutaneously in the left shoulder with 1 mg of WEB emulsified in Freund's complete adjuvant (FCA) to a final volume of 1 ml. SEB in FCA was administered in a similar manner to two other goats (numbers 9122 and 9125). Fourty-two days later, the four animals received a booster injection of the same antigen emulsified in incomplete Freund's adjuvant (IFA) at the right shoulder. By comparison, four goats were immunized by the method of infection and treatment, two (numbers 9113 and 9111) with an infected blood stabilate and two (numbers 9104 and 9120) with 5 ml of supernatant of an infected BUE cell culture. As soon as their rectal temperature reached 40°C, these four goats were treated daily with oxytetracycline (15 mg kg^{-1} day^{-1} intravenously) until their temperature returned to normal. The eight immunized goats and two naive controls were challenged 98 days after the first inoculation by injecting 5 ml of a virulent cell culture supernatant intravenously (Fig. 1).

Experiment 2

Experiment 2 was carried out to confirm the results of the previous experiment. Four out of five goats were inoculated with WEB (numbers 9194, 91100, 91104, 91107) and one with SEB (number 91102). Two injections were given at 94 days interval and all five goats together with two naive controls were challenged 143 days after the first injection (Fig. 1). The dose of antigen and the route of inoculation were similar to those in Experiment 1.

Throughout the experiments, the animals were monitored daily for rectal temperature and symptoms. Serum samples were collected twice a week and preserved at -20°C until use. Brain squash smears (Purchase, 1945) were prepared and stained with Giemsa from any animal which died, to confirm death resulting from heartwater.

Serology

Sera were monitored for the presence of antibody to *Cowdria* by an indirect ELISA method using peroxidase (Martinez et al., 1993a). Sera were tested at 1/400 dilution in PBS 0.1M, pH 7.2 supplemented with 0.1% Tween 20 and 3% skimmed cow milk (blocking buffer). Horseradish peroxidase conjugated rabbit anti-goat immunoglobulin (H+L chains, NORDIK Immunology) diluted 1/6000 in the blocking buffer, was used as second antibody. Positive and negative controls were run in each plate in order to validate the reproducibility of the reaction. In addition, at the peak of the antibody response demonstrated by ELISA, sera were monitored by indirect immunofluorescence with infected BUE cultures as the antigen. Using this technique, anti-BUE antibodies were detected in all goats vaccinated with SEB or WEB in addition to antibodies specific to *Cowdria*. This was due to the presence of cell membrane contaminants in the preparation of elementary body antigens. These anti-cell antibodies were not detected in goats vaccinated by infection and treatment. One serum of each goat collected at the peak of antibody was therefore absorbed on BUE cells to remove antibodies to cell membranes. The percentage of reduction of the absorbance between non-absorbed and absorbed sera was calculated for each goat and used as a correction factor for all points of the serology. Figure 1 represents the ELISA titres plotted after correction.

In vitro seroneutralisation assay

The ability of immune goat sera to inhibit the infection of BUE cells by *Cowdria* was examined with an *in vitro* assay. The immune sera tested were those collected at day 40 post-infection from goats 9111 and 9113, and at day 154 post-infection from all other goats (see Table 2) when the antibody titres averaged a maximum (Fig. 1). In a first set of assays, preimmune and immune sera (inactivated by heating at 56°C for 30 min or not inactivated) were added to a suspension of *Cowdria* at a final concentration of 10% and 0.8 ml of each mixture was added in wells of six-well plates of BUE cells. Positive control wells were inoculated with the suspension of *Cowdria* to which fetal calf serum had been added instead of test serum, non-inoculated wells served as negative controls. Each serum was tested in duplicate. The contact between the cells and the suspension of parasites was allowed to proceed for 2 h at 37°C and 2 ml of fresh culture medium were added to each well. Under our culture conditions, the average time for the appearence of the first plaques is 4.6 to 5 days (Martinez et al., 1993b). The level of infection of cells was thus determined by microscopy at day 4 of culture, before the lysis of infected cells and when the morulae are large enough in the cytoplasm to be easily detected. The cells were stained with eosine-methylene blue (RAL 555 staining kit) and the number of morulae were counted in 30 microscopic fields at 500X magnification. The presence of only one morula per infected cell was verified, and the average number of

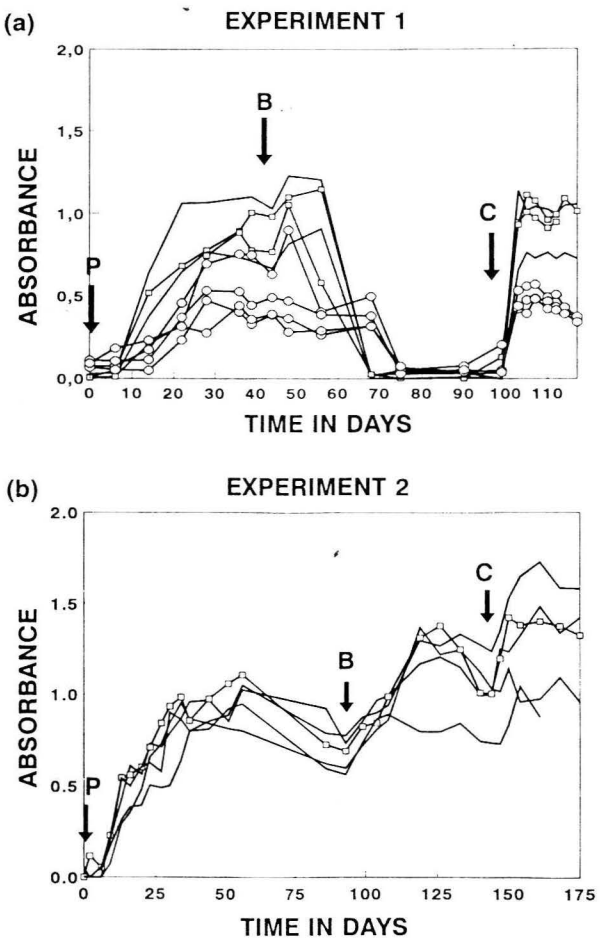


Fig. 1. Evolution of antibody levels measured by ELISA after immunisation of goats by infection and treatment (○) or injection of inactivated elementary bodies of *Cowdria ruminantium* with Freund's adjuvant: whole elementary bodies or WEB (–), sonicated elementary bodies or SEB (□). Dates of injection are indicated by an arrow; (P) priming injection; (B) booster injection; (C) virulent challenge.

morulae per microscopic field was then compared between wells treated with immune or preimmune sera and positive control wells. A second set of assays was performed with the following modifications: (1) the *Cowdria* were preincubated with test sera for 45 minutes at 37°C prior to inoculation of BUE; (2) the cell monolayers were rinsed with medium after the period of contact (2 h) between the parasites and the cells.

Results

In Experiment 1, both goats immunized with WEB became immune and survived the challenge, however, neither of the goats immunized with SEB became protected and both died. In Experiment 2, three out of four goats immunized with WEB became immune and survived the challenge, and the goat immunized with SEB became protected and survived. All four naive control goats (two in each experiment) died after virulent challenge (Table 1).

In both experiments, goats immunized with inactivated *C. ruminantium* did not show any symptom during the course of immunization except a slight hyperthermia in some animals immediately after the injection of antigens in adjuvant. After challenge, goats which proved to be protected, did not show any symptoms of cowdriosis except fever (Table 1). They had a loss of appetite the first 24-48 h of hyperthermia but started eating again rapidly, even before their temperature returned to normal. Although they proved not to be protected against the virulent infection, goats 9122 and 9125 vaccinated with SEB in Experiment I and goat 91100 vaccinated with WEB in Experiment 2, survived significantly longer than the controls. In particular, goats 9122 and 91100 died 24 days and 25 days after challenge, respectively (between 9 and 12 days for control animals), although their rectal temperature had returned to normal and they seemed to be on the path to recovery.

All four goats immunized by infection and treatment in Experiment 1, developed symptoms of cowdriosis the first day of hyperthermia above 40°C. They were then treated daily with oxytetracyclin until their temperature returned to normal (2-3 days). The incubation period for goats immunized by infection and treatment was 14 days when blood was used for infection, and only 6 or 7 days when BUE supernatant was used for infection. All goats recovered after treatment but one of them (number 9104) died suddenly 70 days post-infection while it looked healthy (Table 1). *Cowdria* could not be seen in the brain squash from this goat. The three remaining goats immunized with living *Cowdria* proved solidly immune at challenge; they did not develop any symptom or hyperthermia.

The kinetics of antibody levels detected by ELISA are illustrated in Fig. 1(a) (Experiment 1) and Fig. 1(b) (Experiment 2).

Table 1
Reaction of immunized and control goats injected with a virulent preparation of *Cowdria ruminantium* (Gardel stock).

Immunization procedure[a]	Goat number	Hyperthermia >40°C	Incubation period[b] (days)	Maximum temperature (°C)	Symptoms[c]	Outcome
Experiment 1						
BUE	9104	+	7	40°9	+	death (d70)[d]
BUE	9120	+	6	41°6	+	survival
blood	9113	+	14	40°8	+	survival
blood	9111	+	14	41°1	+	survival
SEB	9122	+	9	40°7	+	death (d24)
SEB	9125	+	7	41°4	+	death (d14)
WEB	9124	+	10	41°5	_	survival
WEB	9126	+	7	41°2	_	survival
naive	9138	+	5	40°2	+	death (d11)
naive	9141	+	8	40°6	+	death (d12)
Experiment 2						
SEB	91102	+	7	41°2	_	survival
WEB	9194	+	7	41°8	_	survival
WEB	91100	+	7	41°5	+	death (d25)
WEB	91104	+	7	41°2	_	survival
WEB	91107	+	7	41°6	_	survival
naive	9149	+	8	40°0	+	death (d9)
naive	91103	+	7	41°1	+	death (d9)

[a] Goats were immunized by intravenous injection of blood or bovine endothelial cells (BUE) infected with *C. ruminantium* followed by treatment with oxytetracyclin. Alternatively goats were immunized by two injections of inactivated intact (WEB) or sonicated (SEB) elementary bodies of *Cowdria* in Freund's adjuvant. All animals were challenged by IV injection of *in vitro* cultivated *C. ruminantium*.
[b] Number of days between injection and hyperthermia.
[c] Symptoms other than hyperthermia : recumbency, opisthotonus.
[d] Days after injection of *Cowdria*.

In Experiment 1, specific antibodies levels were detected in serum between 1 and 2 weeks post-inoculation of goats with WEB or SEB, and between 1 and 3 weeks after

inoculation with virulent blood or BUE culture. The antibody levels reached a plateau which lasted until days 55-65 post-inoculation when all animals became sero-negative again whatever the immunization procedure. They remained sero-negative until challenge which induced a rapid augmentation of their antibody level. The antibody levels of goats were similar within each group vaccinated with WEB, SEB, blood or BUE whether they survived or not, excepted for goat 9122, whith an intermediate antibody level (Fig. 1(a)). In addition, it should be noted that despite the lower titre of antibody elicited by immunization by infection and treatment, this method proved to be the best since none of the goats developed hyperthermia following challenge, in contrast to the goats immunized by injection of inactivated *Cowdria*.

Table 2
Results of the effect of immune sera on the neutralization of the infection of BUE cells by *Cowdria ruminantium in vitro*.

	presence of *Cowdria* in BUE cells				
Goat number	Negative control	Positive control	Preimmune serum	Immune serum	Immune serum decomplemented
9113[a]	-	20±5	20±7	15±5	17±4
9111[a]	-	23±9	17±4	15±5	10±4
9113[b]	-	+	+	+	+
9111[b]	-	+	+	+	+
9124[b]	-	+	+	+	+
9126[b]	-	+	+	+	+
9194[b]	-	+	+	+	+
91102[b]	-	+	+	+	+
91104[b]	-	+	+	+	+
91107[b]	-	+	+	+	+

[a] The inoculum of *Cowdria* added with 10% of test serum was put into contact with BUE cells for 2 hours and fresh medium added without removing the inoculum. The results are expressed in number of morulae per microscopic field (500X magnification).
[b] The parasite was preincubated with the serum (10% final dilution) for 45 min at 37°C before inoculation of the cells and the cell monolayer was rinsed with medium after 2 hours of contact with the inoculum. The number of morulae per microscopic field averaged 1 in this set of assays and the results were therefore only expressed by + (presence) or - (absence) of morulae in the cells.

In Experiment 2, antibodies were detectable between 1 and 2 weeks post-infection and their level reached a plateau after 3-4 weeks (Fig. 1(b)). In contrast to Experiment 1, goats remained sero-positive throughtout the experiment. Finally, there was no significant difference in antibody levels between the goat which died and the four goats which survived.

Under our experimental conditions, none of the sera tested neutralized or significantly decreased the infection of BUE cells by *C. ruminantium*, whether they were collected from goats immunized by either inactivated elementary bodies or by infection and treatment. The inactivation of serum complement proteins had no effect on the neutralization of infectivity (Table 2).

Discussion

This is the first report of a successful protection of ruminants against heartwater acquired after immunization with inactivated antigens of *C. ruminantium*. We did not succeed in protecting 100% of the goats. However, the challenge was severe, as the concentration of *Cowdria* in culture supernatants used for the challenge was far higher than in a natural tick infection. Thus, the incubation period was reduced from 14 days to an average of 7 days when goats were injected with infected blood and *C. ruminantium* grown in BUE, respectively (Table 1). This is in agreement with the observations of several authors that the course of the disease is affected by the dose of the infection (Alexander, 1931; Bezuidenhout, 1981).

Because of the intracellular status of this parasite, it was a common belief that immunity to heartwater was cell mediated. In a mouse model of cowdriosis, Du Plessis et al. (1991) showed that immune spleen cells depleted of Lyt-2^+ T-cells ($CD8^+$ cells) were unable to confer resistance to challenge with *C. ruminantium* to recipient mice, whereas the depletion of $L3T4^+$ ($CD4^+$ cells) had no effect on this protection. In our study, the parasites were inactivated and therefore unable to proliferate in endothelial cells. Thus, it is unlikely that *Cowdria* antigens were presented in association with MHC class I molecules to $CD8^+$ lymphocytes to elicit a cytotoxic response. This suggests that a good T-helper response might have been essential in conferring protection to the disease and that more antibodies alone did not play a major role. Indeed, in our study, the antibody levels were similar between protected and non-protected animals throughout the course of immunization (Fig. 1). Moreover, although the study on *in vitro* seroneutralisation assay was rather limited and the test was not standardized, it was shown that immune sera with or without complement were unable to inhibit or significantly decrease the infection of BUE cells by *Cowdria* (Table 2). Similarly, several authors failed to protect sheep against heartwater by transfer of serum or IgG purified from immune animals whether given at the time of infection or during the incubation period (Alexander, 1931; Du Plessis, 1970). In addition, the observation of low antibody titres is a common observation on goats solidly immune after vaccination by infection and treatment or

by injection of an attenuated stock of *C. ruminantium* (Senegal) (D. Martinez, unpublished data, 1992), indicating, in agreement with Du Plessis et al. (1984), that protection is not correlated with the level of antibody in plasma. However, the involvement of specific antibodies in antibody-dependent cytotoxicity reactions or opsonization were not investigated and therefore cannot be completely excluded. Immunological mechanisms which induced protection in goats against cowdriosis when using killed elementary bodies are currently under investigation. However, recent studies *in vitro* have shown that the development of *C. ruminantium* in endothelial cells can be prevented by α and γ interferons (Totté et al., 1993). Therefore, the possible involvement of immune T-cells (Th subsets) released cytokines in mediating protection against *C. ruminantium in vivo* seems to be likely. Nonetheless, the experiments showing that a substantial degree of protection can be achieved by immunizing goats with dead *C. ruminantium* is of great value in the search of recombinant antigens which will be the basis for new safe vaccines.

Acknowledgements

This work was supported by a grant from the European Community (Directorate General XII) under contract N° TS3-CT91-0007.

References

1. Alexander, R.A., 1931. Heartwater. The present state of our knowledge of the disease. 17th report of the director of veterinary services and animal industry, Union of South Africa, 89-149.

2. Barré, N., Uilenberg, G., Morel, P.C. and Camus, E., 1987. Danger of introducing heartwater onto the American mainland: Potential role of indigenous and exotic *Amblyomma* ticks. *Onderstepoort J. Vet. Res.* 54, 405-417.

3. Bezuidenhout, J.D., 1981. The development of a new heartwater vaccine, using *Amblyomma hebraeum* nymphs infected with *Cowdria ruminantium*, p. 41-45. in G.B. Whitehead and J.D. Gibson (ed.), Proc. Int. Congr. on Tick Biology and Control, Tick Research Unit, Rhodes University, Grahamston, South Africa.

4. Bezuidenhout, J.D., Paterson, C.L. and Barnard, B.J.D., 1985. *In vitro* cultivation of *Cowdria ruminantium*. *Onderstepoort J. Vet. Res.* 52, 113-120.

5. Bezuidenhout, J.D., 1989. *Cowdria* vaccines, p. 31-42. In I.G. Wright, Veterinary protozoan and hemoparasite vaccines. Boca Raton, CRC Press, Inc.

6. Birnie, E.L., Burridge, M., Camus, E., and Barré, N., 1984. Heartwater in the Caribbean, isolation of *Cowdria ruminantium* from Antigua. *Vet. Rec.* 116, 121-123.

7. Bradford, M.M., 1976. Microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Chem.* 72, 248-254.

8. Du Plessis, J.L., 1970. Immunity to heartwater. I. A preliminary note on the role of serum antibodies. *Onderstepoort J. Vet. Res.* 37, 147-150.

9. Du Plessis, J.L., Bezuidenhout, J.D. and Lüdemann, C.J.F., 1984. The immunization of calves against heartwater: subsequent immunity both in the absence and presence of natural tick challenge. *Onderstepoort J. Vet. Res.* 51, 193-196.

10. Du Plessis, J.L., Van Gas, L., Olivier, J.A. and Bezuidenhout, J.D., 1989. The heterogeneity of *Cowdria ruminantium* stocks, cross-immunity and serology in sheep and pathogenicity to mice. *Onderstepoort J. Vet. Res.* 56, 195-201.

11. Du Plessis, J.L., Berche, P. and Van Gas, L., 1991. T Cell-mediated immunity to *Cowdria ruminantium* in mice: the protective role of Lyt-2^+ T cells. *Onderstepoort J. Vet. Res.* 58, 171-179.

12. Flach, E.J., Woodford, J.D., Morzaria, S.P., Dolan, T.T. and Shambwana, I., 1990. Identification of *Babesia bovis* and *Cowdria ruminantium* on the island of Unguja, Zanzibar. *Vet. Rec.* 126, 57-59.

13. Jongejan, F., 1991. Protective immunity to heartwater (*Cowdria ruminantium* infection) is acquired after vaccination with *in vitro* attenuated rickettsiae. *Infec. Immun.* 59, 729-731.

14. Jongejan, F. and Thielemans, M.J.C., 1991. Antigenic diversity of *Cowdria ruminantium* determined by cross-immunity. *Res. Vet. Sci.* 51, 24-28.

15. Logan, L.L., Whyard, T.C., Quintero, J.C. and Mebus, C.A., 1987. The development of *Cowdria ruminantium* in neutrophils. *Onderstepoort J. Vet. Res.* 54, 197-204.

16. Martinez, D., Swinkels, J., Camus, E. and Jongejan, F., 1990. Comparaison de trois antigènes pour le diagnostic de la cowdriose par immunofluorescence indirecte. *Rev. Elev. Méd. Vét. Pays Trop.* 43, 159-166.

17. Martinez, D., Coisne. S., Sheikboudou,.C. and Jongejan, F., 1993a. Detection of antibodies to *Cowdria ruminantium* in the serum of domestic ruminants by ELISA. *Rev. Elev. Méd. Vét. Pays Trop.* 46, 115-120.

18. Martinez, D., Sheikboudou, C., Couraud, P.O. and Bensaid, A., 1993b. *In vitro* infection of bovine brain endothelial cells by *Cowdria ruminantium*. *Res. Vet. Sci.* 55, 258-260.

19. Perreau, P., P.C. Morel, N. Barré, and P. Durand. 1980. Existence de la cowdriose (heartwater) à *Cowdria ruminantium* chez les ruminants des Antilles françaises (La Guadeloupe) et des Mascareignes (La Réunion et île Maurice). *Rev. Elev. Méd. Vét. Pays Trop.* 33, 21-22.

20. Provost, A. and Bezuidenhout, J.D., 1987. The historical background and global importance of heartwater. *Onderstepoort J. Vet. res.* 54, 165-169.

21. Purchase, H.S., 1945. A simple and rapid method for demonstrating *Rickettsia ruminantium* (Cowdry, 1925), in heartwater brains. *Vet. Rec.* 57, 413-414.

22. Totté, P., Blankaert, D., Zilimwabagabo, P. and Werenne, J., 1993. Inhibition of infectious yield of *Cowdria ruminantium* by interferons alpha and gamma in endothelial cells. *Rev. Elev. Méd. Vét. Pays Trop.* 46, 189-194.

23. Uilenberg, G., 1983. Heartwater (*Cowdria ruminantium* infection): Current status. *Adv. vet. Sci. comp. Med.* 27, 427-480.

24. Uilenberg, G., Barré, N., Camus, E., Burridge, M.J. and Garris, G.I., 1984. Heartwater in the Caribbean. *Prev. Vet. Med.* 2, 255-267.

25. Uilenberg, G., Camus, E. and Barré, N., 1985. Quelques observations sur une souche de *Cowdria ruminantium* isolée en Guadeloupe (Antilles françaises). *Rev. Elev. Méd. Vét. Pays Trop.* 38, 34-42.

CHAPTER 4

Infection of bovine brain microvessel endothelial cells with *Cowdria ruminantium* elicits IL-1β, -6, and -8 mRNA production and expression of an unusual MHC Class II DQα transcript

S. Bourdoulous[a], A. Bensaid[b], D. Martinez[c], C. Sheikboudou[c], I. Trap[b], A. D. Strosberg[a], P.O. Couraud[a].

a Molecular Immunopharmacology, CNRS UPR 0415, Cochin Institute for Molecular Genetics, Paris, France.

b CIRAD-EMVT, 10 rue Pierre Curie, 94704 Maisons-Alfort Cedex, France.

c Centre International en Recherche Agronomique pour le Développement, Département d'Elevage et de Médecine Vétérinaire des Pays Tropicaux (CIRAD-EMVT), BP 1232, 97184 Pointe à Pitre Cedex, Guadeloupe, West Indies.

The Journal of Immunology, 1995, 154, 4032-4038.

Abstract

Cowdria ruminantium is a bacterial parasite that infects ruminants, causing an acute and often fatal disease. These obligate intracellular Gram-negative bacteria preferentially infect neutrophils and vascular endothelial cells, especially in the brain. The present study was performed with bovine brain microvessel endothelial cells in culture, infected by *C. ruminantium* in the presence or absence of IFNγ. Infection induced the production of IL-1β, -6 and -8 mRNAs and this effect was potentiated by IFNγ. A semi-quantitative PCR analysis indicated that similar amounts of IL-1β and IL-6 mRNAs were produced in response to *C. ruminantium* infection and to treatment with 30-40 ng ml^{-1} LPS. In addition, whereas IFNγ induced the synthesis of a MHC class II DQα transcript (1.3 kb), an unusual transcript (1.5 kb) was induced by infection and not after LPS treatment. Infection did not affect MHC class I, class II DQβ, and invariant chain mRNA levels. The present results suggest that *C. ruminantium* infection raises the immune activity of brain endothelial cells *in vitro* and that only part of this response may be attributed to LPS. One can hypothesize that cerebral endothelium *in vivo* could efficiently contribute, by MHC antigen expression and production of interleukins, to the activation and/or recruitment of leukocytes to the brain and thus play an active role in the pathogenesis of cowdriosis and in the immune response to this pathogen.

Introduction

Infection by intracellular pathogens is essentially controlled by T cell-mediated immunity. Immune responses to organisms infecting primarily mononuclear phagocytes (e.g., *Mycobacterium tuberculosis*, or *Listeria monocytogenes*) were initially considered to be only dependent on the capacity of infected macrophages to express MHC class II molecules and present microbial antigens to CD4+ T cells (Zinkernagel and Doherty, 1979). On the other hand, host cells infected by other intracellular pathogens were thought to be mainly killed by MHC class I-restricted CD8+ T cells (Kaufman, 1988). More recent data support the notion that T cell-mediated immunity against most intracellular pathogens involves both T cell subsets, CD4+ and CD8+, although with various relative contributions, depending on the pathogen (Kaufman, 1988; Gazzinelli et al., 1991).

C. ruminantium is a tick-borne bacterial parasite that infects both wild and domestic ruminants, causing an acute and often fatal disease known as Heartwater (or Cowdriosis), which is characterized by hydropericardium, fever, and signs of central nervous system disorder (Prozesky, 1987). Together with East Coast Fever, caused by *Theileria parva* (in East and Central Africa), heartwater is a disease with a considerable economic impact throughout Sub-Saharan Africa and the Caribbean islands (Norval et al., 1992). *C. ruminantium* is an obligate intracellular Gram-negative bacteria, with a taxonomic position near that of *Ehrlichia* and *Chlamydia* (Van Vliet et al., 1992), that preferentially infects vascular endothelial cells and neutrophils: presence of organisms in brain capillary endothelial cells appears as a constant feature and a major diagnosis criterion of the disease (Camus and Barré, 1988).

Vaccination trials against *C. ruminantium* have been initiated in several laboratories. To overcome the fastiduous and dangerous method of infection and treatment of ruminants, these trials were based on injection of *in vitro* attenuated organisms (Jongejan, 1991) or, more recently, of killed elementary bodies (Martinez et al., 1994). Despite some success, it is clear that any wide vaccination program is hampered by the antigenic variability among the different *C. ruminantium* strains (Jongejan and Thielemans, 1991) and, more importantly, by the limited current knowledge of the host immune response to this pathogen.

Mechanisms conferring protection to the immunized animals are still poorly understood. A humoral response has been detected in infected ruminants, but does not seem to participate to their protection against the pathogen (Prozesky, 1987). Together with the report of a T cell-mediated immune response in experimentally infected mice (Du Plessis et al., 1991), this observation supports T cell-mediated immunity in ruminants. Because conventional antigen-presenting cells (APC) are generally not infected by *C. ruminantium*, we questioned whether brain capillary endothelial cells could contribute to the development of cellular immunity through cytokine production and MHC molecule expression. We initiated the present study with bovine brain microvessel endothelial cells (BBEC), infected *in vitro* with

C. ruminantium as described recently (Martinez et al., 1993). These cells have been extensively characterized in our laboratory (Durieu-Trautmann et al., 1991; Coutinho et al., 1991; Bourdoulous et al., 1993). (1) They maintain in culture a fully differentiated phenotype and express several blood-brain barrier markers, including numerous tight junctions. (2) They are inducible by IFN-γ for expression of MHC class I and class II molecules and of the non-polymorphic invariant chain (Ii), which is involved in the MHC class II intracellular trafficking. Our study establishes that *C. ruminantium* infection of BBEC cells elicits the accumulation of IL-1β, IL-6 and IL-8 mRNAs as well as the expression of an unusual MHC class II DQα transcript.

Materials and methods

Chemicals

Bovine rIFN-γ, was a kind gift of Ciba-Geigy (Basel, Switzerland). *Escherichia coli* LPS was purchased from Sigma Chemical Co. (St Louis, MO)

Cell culture

BBEC were isolated from bovine brain cortex and grown in culture as described previously (Durieu-Trautmann et al., 1991). They were passed once a week after trypsin/EDTA treatment (0.05% / 0.02%) and seeded at a density of 10^4 cells/cm^2 on gelatin-coated dishes in DMEM (1 g l^{-1} glucose; Seromed, France), supplemented with 2 mM glutamine, 10 % heat-inactivated fetal calf serum and 1 ng ml^{-1} basic Fibroblast Growth Factor (Boehringer Mannheim, Germany). Medium was changed every 3 to 4 days. In these conditions, confluence was typically reached after 4 days in culture, corresponding to a density of 1.2 to 1.5 x 10^5 cells/cm2. In the experiments described below, BBEC were used between the 5th and 11th passage.

BBEC infection by Cowdria ruminantium

Infections by *C. ruminantium* were initiated on confluent monolayers in culture medium supplemented with 2.95 mg ml^{-1} tryptose phosphate broth (Difco, Detroit, USA). BBEC were first inoculated with the supernatant of bovine umbilical endothelial cells, originally infected by *C. ruminantium* from the blood of an infected goat (Martinez et al., 1993). When a strong cytopathogenic effect appeared, supernatants of infected cells were collected and transferred to new cultures. After incubation for 2 h at 37°C, fresh medium was added; cell lysis usually took place 4

days after infection and then spreaded to the rest of the monolayer in a few days. When about 80-90% of the monolayer was lysed, the supernatant was used to infect new cultures.

Cell treatments

When indicated, cells were treated with IFN-γ (1 U ml^{-1}) from day 0 of infection. Medium, supplemented with the same concentration of IFN-γ, was changed every day untill cell harvesting for RNA preparation. Alternatively, uninfected cells were treated with LPS (1 ng ml^{-1} to 10 mg ml^{-1}).

cDNA probes

The HLA class II locus-specific probes were the generous gift of Dr. D. Piatier-Tonneau (Institut d'Embryologie, Nogent-sur-Marne, France). The human DQα probe is a 500-bp restriction fragment of pDCH1, subcloned into the *Sma*I-*Sac*I sites of pUC12. The human DRα probe is a 700-bp restriction fragment from pDRH-7, subcloned into pUC9 (Piatier-Tonneau et al., 1986). The Ii probe is a 650-bp insert in the *Bam*HI site of pCMU IV, containing the complete sequence encoding the human p43 chain (Lotteau et al., 1990), kindly provided by Dr V. Lotteau (Institut des Cordeliers, Paris, France). The bovine class I locus-specific probes, BoLA A and B, are *Pst*I-*Eco*RI 240-bp restriction fragment from a 1400-pb cDNA subcloned into *Eco*RI site of pUC19 (Bensaid et al., 1991). The HLA DRβ and DQβ cDNAs were a generous gift of Dr. L. Anderson (Uppsala University, Sweden). Extensive cross-hybridization of human MHC class II cDNAs to bovine genes (BoLA) have been widely described (Andersson and Rask, 1988; Van der Poel et al., 1990)

RNA isolation and Northern blot analysis

RNA preparations were performed 2 to 5 days post-infection, before (days 2 and 3) or after (days 4 and 5) cell lysis appeared. The cell monolayers were lysed in guanidinium isothiocyanate and RNA isolated by phenol-chloroform extraction and precipitation with isopropanol as previously described (Chomczynski and Sacchi, 1987). Samples (10 µg) of total cellular RNA were denatured with formaldehyde for 10 min at 65°C, and RNA was size-fractionated by electrophoresis through a 1.2% agarose gel containing ethidium bromide before transfer to nylon membrane (Hybond N+, Amersham, Les Ulis, France) in 0.05 M NaOH. Homogeneity of the RNA amounts loaded on the gel was checked by UV staining of the 18S and 28S

rRNA bands on the gel and the membrane.

For the HLA class II DQα, DRα, DQβ and DRβ probes, prehybridization was performed at 42°C in a solution containing 40% deionized formamide, 4X SSC, 5X Denhardt's solution, 8 mM Tris pH 7.5, 50 mM phosphate buffer, pH 6.5, 0.2% SDS, and 100 μg ml^{-1} of denatured salmon sperm DNA for 6 to 8h. Hybridization was carried out at 42°C overnight in the same solution containing 10% dextran sulfate and denatured ^{32}P-labeled probes (10^6 cpm μg^{-1}). Blots were then washed four times for 30 min at 50°C (final wash: 0.1X SSC + 0.1% SDS).

For hybridization with the BoLA class I A and B probes and the human Ii probe, blots were prehybridized for 6 to 8 h at 65°C in solution containing 1% BSA fraction V, 1 mM EDTA, 0.5 M phosphate buffer pH 7.2, 7% SDS, and 100 μg ml^{-1} of denatured salmon sperm DNA. Hybridization was performed overnight at 65°C in the same solution in the presence of denatured ^{32}P-labeled probes. The final wash was in 1X SSC + 0.1% SDS at 65°C for Ii-hybridized blots, in 0.1X SSC + 0.25% SDS at 68°C for BoLA-hybridized blots. Blots were then exposed to XAR-5 films (Eastman Kodak, Rochester, NY) with intensifying screens at -80°C.

PCR

For PCR analysis of BoLA DQα transcripts, a sense (5'-TCCCTCTGGCTACTATACCCA-3') primer and an anti-sense (5'-CAGACGCGAGTCCACCGGGGA-3') were synthesized corresponding to nucleotides 402-423 and 1683-1704, respectively, of the bovine MHC class II DQα gene (Van der Poel et al., 1990).

Primers for the bovine IL1-β cDNA amplification were deduced from the published sequence (Leong et al., 1988): the sense primer, corresponding to nucleotides 501-524 (5'-TGGTGTTCTGCATGAGCTTTGTG-3') and the anti-sense primer corresponding to nucleotides 823-845 (5'-TTAGGGAGAGAGGGTTTCCATTC-3'). For the bovine IL-6 cDNA amplification, a sense (5'- ATGAACTCCCGCTTCACAAGCG-3') and an anti-sense primer (5'- CAAGGTTTCTCAGGATGAGGAT-3') were synthesized according to the available sequence (Droogmans et al., 1992), corresponding to nucleotides 43-64 and 604-625, respectively. Sequence data for bovine IL-8 is not available and primers for mRNA amplification of IL-8 were deduced by comparing the human and porcine IL-8 mRNA sequence (Mukaida et al., 1989). After selecting portions highly homologous between these two mRNA species, a forward primer (5'-TTGGCAGCCTTCCTGATTTCTGCA-3') and a reverse primer (5'-AACCCTGCACCCAGTTTTCCTT-3') were synthetized. As an internal standard of reverse transcriptase efficiency, a set of primers specific for the human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were purchased from Clontech (Palo Alto, CA). All pairs of primers hybridize to different exons, allowing

detection of contaminating genomic DNA in the cDNA preparations. In addition, negative controls performed with water in place of cDNAs were included in each experiment in order to exclude potential PCR artifacts caused by contamination of samples.

First strand cDNA synthesis was performed on 3 to 5 μg of total RNA using an oligo(dT) primer and MMLV reverse transcriptase, according to the manufacturer's instructions (Life Technologies, Bethesda, USA). cDNA (250 ng) was subjected to 30 cycles of amplification (93°C, 1 min; 55°C, 1.5 min; 72°C, 1.5 min) using 2.5 units of *Thermophylus aquaticus* polymerase (Perkin Elmer Cetus, Norwalk, CT) in 50 μl of a buffer consisting of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg ml^{-1} gelatin, 250 μM each deoxynucleotide triphosphate, and 250 nM each primer. For IL-8 amplification, 1 μl of each sample was submitted to 10 additional cycles of PCR in fresh PCR buffer. The PCR-amplified fragments were electrophoresed through a 2% agarose gel. Size markers (MW) used are the 123-bp DNA ladder (Life Technologies). Densitometry scanning of the amplified products was performed using National Institute of Health Image 1.44 program.

DQα amplification products were electrophoresed through a gel and blotted onto nylon membrane which was then hybridized with human DQα probe.

Direct DNA sequencing

The direct sequencing of the PCR-amplified fragments was performed as described previously (Akli et al., 1991)

Results

Induction of cytokine mRNA after C.Ruminantium infection

Expression of cytokine mRNA in BBECs was analyzed by reverse transcription PCR early after infection (6 h), using G3PDH as an internal standard for reverse transcriptase efficiency. Results presented in Fig. 1 indicate that *C. ruminantium* induced the expression of IL-1β and IL-6 mRNAs: although no signal was detected in uninfected cells, amplified products of the expected size were obtained after infection, and their identity was further confirmed by the presence of several restriction sites predicted from the corresponding nucleotide sequences.

To compare the magnitude of the cytokine response of BBECs to infection with their response to LPS, we performed a semi-quantitative analysis of mRNA levels in the presence of a wide range of LPS concentrations. As shown in Fig. 1, IL-1β and IL-6 signals were detected after 6 h of treatment with 10 ng ml^{-1} LPS, the

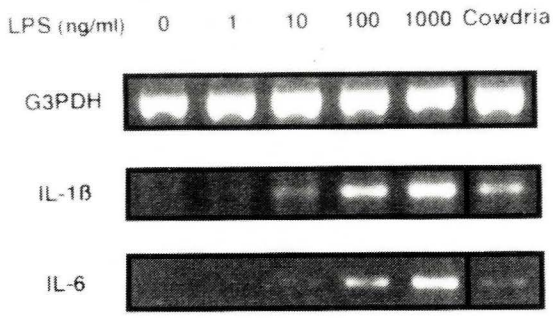


Fig. 1. Semi-quantitative PCR analysis of IL-1β and IL-6 mRNAs in BBEC after 6 h of *C. ruminantium* infection or stimulation by LPS (0-1000 ng ml^{-1}). Reverse transcriptase efficiency of mRNA was assessed by G3PDH amplification for each sample.

intensity of the signals increasing with LPS concentrations, up to 1 mg ml^{-1}. Densitometry scanning of the amplified fragments, normalized to G3PDH signal intensity, revealed that *C. ruminantium* infection induced IL-1β and IL-6 mRNAs at levels comparable with those induced by 30 to 40 ng ml^{-1} LPS. In the same conditions, no IL-8 signal was observed following infection.

To investigate whether the expression of cytokine mRNAs was maintained during bacterial infection until cell lysis, BBEC response to *C.Ruminantium* was further analyzed at day 3 post-infection (1 day before cell lysis), in the absence or presence of IFN-γ (1 U ml^{-1}). Results presented in Fig. 2 indicate that *C. ruminantium*, as well as IFN-γ, induced the expression of IL-1β mRNA. IL-6 and IL-8 mRNAs were also detected in infected BBEC, but were not significantly induced by IFN-γ treatment. When cells were infected in the presence of IFN-γ, stronger IL-1β, IL-6 and IL-8 signals were observed, which might reflect a synergy between IFN-γ and *C. ruminantium*.

These results indicate that *C. ruminantium* infection induces an early and sustained production, potentiated by IFNγ- stimulation, of IL-1β and IL-6 mRNAs by BBEC, as well as a later expression of IL-8 mRNA, and suggest that LPS mediate this response.

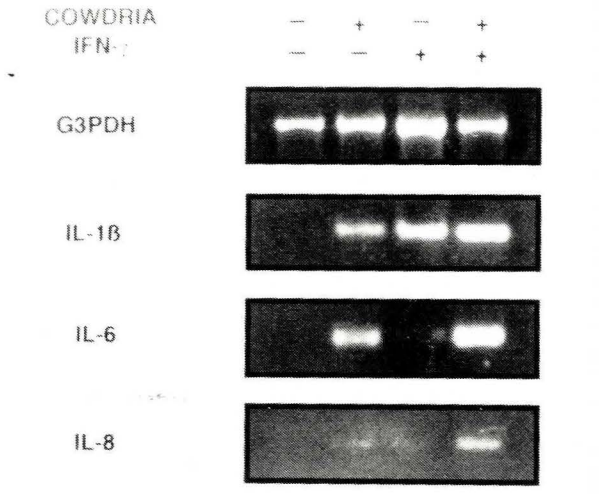


Fig. 2. PCR analysis of IL-1β, IL-6 and IL-8 mRNAs in BBEC, untreated or after 3 days of treatment by IFN-γ (1 U ml^{-1}) and/or infection by *C. ruminantium*. Reverse transcriptase efficiency of mRNA was assessed by G3PDH amplification for each sample.

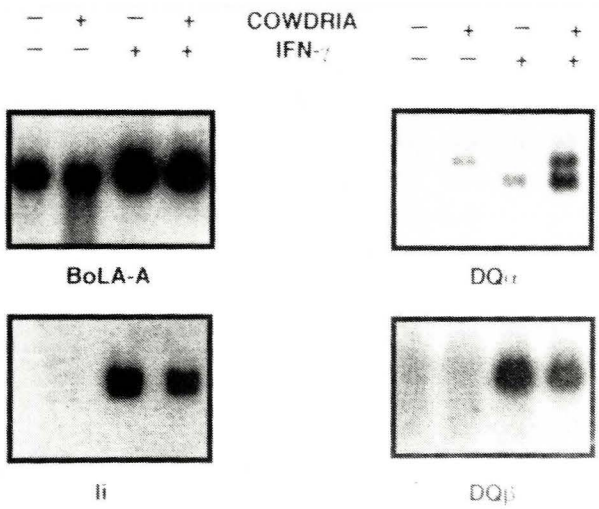


Fig. 3. Northern blot analysis of MHC class I BoLA-A, MHC class II DQα and DQβ and invariant chain Ii mRNA, as indicated, in BBEC, untreated or after 3 days of treatment by IFN-γ (1 U ml^{-1}) and/or infection by *C. ruminantium*.

Northern blot analysis of MHC class I, class II, and Ii mRNA levels after C. ruminantium infection

MHC class I BoLA A and B, class II DQα and DQβ, as well as Ii mRNA steady-state levels were measured on BBEC, uninfected or at day 3 post-infection, in the absence or presence of IFN-g (1 U ml^{-1}). As shown in Fig. 3, the constitutive level of MHC class I BoLA A mRNA was elevated after treatment with IFN-γ but was not affected by *C. ruminantium* infection. Similar results were obtained by hybridization with BoLA B probe (not shown). IFN-γ induced transcription of MHC class II DQα, DQβ and Ii genes, as reported previously (Coutinho et al., 1991; Bourdoulous et al., 1993). DQβ and Ii mRNA levels did not change after infection (Fig. 3). In addition, MHC class II DRα and DRβ mRNAs were also induced after IFN-γ treatment but were not affected by *C. ruminantium* infection (and not shown).

On the contrary, *C. ruminantium* infection selectively elicited the expression of a DQα probe-hybridizing transcript. Interestingly, this 1.5-kb transcript appeared clearly distinct from the 1.3-kb IFN-γ-induced DQα transcript, and both transcripts were detected when BBEC were infected in the presence of IFN-γ (Fig. 3). Similar kinetics of expression of these transcripts were observed, hybridization signals being detected from 1 to 4 days post infection or IFN-γ treatment (Fig. 4). However, earlier expression of the infection-induced transcript was observed in the presence of IFN-γ: the 1.5-kb signal was detectable as soon as 6 h post-infection, and was maximal after 24 h. The kinetics of expression of the IFN-γ-induced 1.3 kb transcript was not affected by infection (Fig. 4).

High concentrations of LPS (10 μg ml^{-1}) did not induce MHC class II DQα mRNA, but completely inhibited the induction by IFN-γ (Fig. 5); this latter effect was not observed with lower concentrations (10 ng ml^{-1}) of LPS. These results strongly suggest that the up-regulation of DQα mRNA by *C. ruminantium* infection is mediated by a LPS-independent mechanism.

Identification of the infection-induced DQα hybridizing transcript

Northern blot analysis of DQα mRNA following *C. ruminantium* infection was confirmed by reverse transcription PCR analysis, (Fig. 6A). Using DQα-specific primers corresponding to sequences in exons 2 and 4, a fragment of the expected size (614 bp), encompassing most of the coding sequence, was amplified from RNA samples prepared with IFN-γ-treated cells. An amplification product of the same size was also observed with samples from *C. ruminantium*-infected cells. As control, no amplified fragment was visible with RNA from untreated uninfected cells, or from genomic DNA (Fig. 6, lane G). Hybridization with the DQα probe

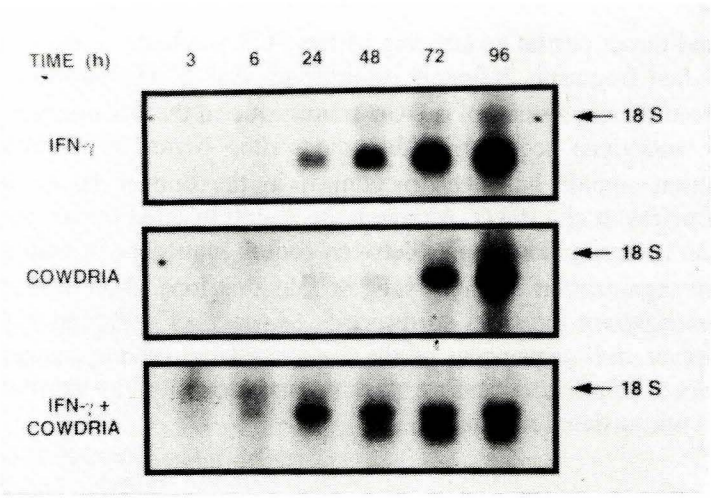


Fig. 4. Kinetics of expression of MHC class II DQα mRNA in BBEC, in response to IFN-γ (1 U ml^{-1}) and/or infection by *C. ruminantium*. Hybridization was performed using human DQα probe. 18S ribosomal RNA is arrowed.

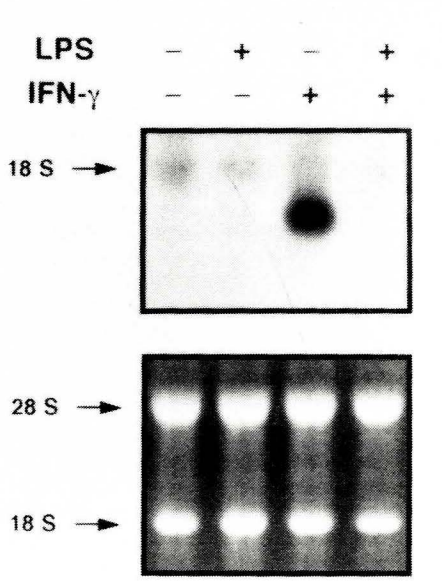


Fig. 5. (top) Northern blot analysis of MHC class II DQα mRNA in BBEC untreated or treated for 3 days by IFN-γ (1 U ml^{-1}) and/or LPS (10 μg ml^{-1}).
(bottom) UV staining of the same blot before hybridization. 18S and 28S ribosomal RNAs are arrowed.

(Fig. 6B) and direct partial sequencing of the PCR products confirmed the identity of the amplified fragment, definitely establishing that *C. ruminantium* infection of BBEC induced the expression of a DQα transcript and that its unusual size was not due to any additional sequence within the coding region. The presence of two polyadenylation signals has been mentioned in the human HLA DQα primary sequence (Auffray et al., 1987). Although the 3'-untranslated bovine sequence is not available, the extensive homologies between coding sequences in both species and a similar gene organization strongly suggest that this long DQα transcript induced after *C. ruminantium* infection corresponds to usage of a second polyadenylation signal. However, full sequencing of the 3'-untranslated bovine sequence would be required before definitely excluding other possibilities, such as alternate splicing in the 3'- or 5'- untranslated regions.

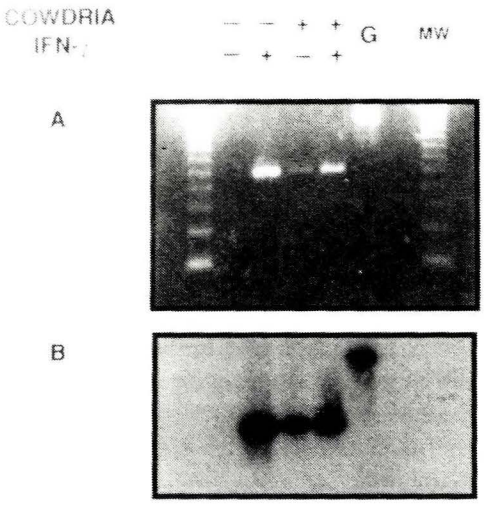


Fig. 6. (A) PCR analysis of DQα mRNA in BBEC, untreated or after 3 days of treatment by IFN-γ (1 U ml^{-1}) and/or infection by *C. ruminantium*. Fragments of the expected size, 614 bp from RNA samples and 1.3 kb from genomic sample (G), were amplified. Size markers (MW) are the 123-bp DNA ladder. (B) The gel was blotted on nylon membrane and hybridized with human DQα probe.

Discussion

This study demonstrates that *C. ruminantium* infection of BBEC resulted in sustained synthesis of IL-1β, IL-6 and IL-8 mRNA up to three days post-infection, just before cell lysis. In addition, whereas MHC class I, class II DRα, DRβ, DQβ, and Ii remained unaffected following infection, MHC class II DQα mRNA was induced. This 1.5-kb transcript, unambiguously identified by PCR analysis and partial sequencing, is clearly different from the IFN-γ-induced form (1.3 kb). Despite the presence of two polyadenylation signals in the human HLA DQα primary sequence, mostly short transcripts corresponding to the first one have been observed so far, the second polyadenylation signal being apparently used only when the first one was altered by somatic mutations (Auffray et al., 1987). Our data strongly suggest that the long DQα transcript induced after *C. ruminantium* infection corresponds to usage of the second polyadenylation signal. This would constitute the first example, to our knowledge, of the specific and differential induction of the two DQα transcripts by different stimuli, IFN-γ and bacterial infection, respectively. The functional relevance of this finding remains to be established.

Exogenous microbial antigens are known to be processed by phagocytes and presented to $CD4^+$ T cells in the context of MHC class II molecules. However, some particulate bacterial antigens have been shown recently to be unusually processed and presented to $CD8^+$ T cells in association with MHC class I molecules (Carbone and Bevan, 1990; Pfeifer et al., 1993). These data are in good agreement with observations that both $CD4^+$ and $CD8^+$ T cell subsets are implicated, to various degrees, in the cell-mediated immune response to a variety of intracellular pathogens. Although $CD4^+$ T cells appeared to be most important for immunity to *Salmonella typhimurium* or *Escherichia coli*, with only a minor contribution from the CD8+ subset (Nauciel, 1990), a synergistic role of both subsets has been reported in immunity to *Toxoplasma gondii* (Gazzinelli et al., 1991). In the first case, a strong correlation has been documented between the induction of MHC class II expression on macrophages and the generation of the antigen-specific $CD4^+$ T cell response. Regarding brain microvascular endothelial cells, we have shown previously that MHC class II expression on BBEC cells, although not constitutive, can be induced by treatment with IFN-γ (14, 15); moreover, the present study shows that MHC class II DQα gene transcription is directly up-regulated by *C. ruminantium* infection. From these data, it is tempting to speculate that MHC class II expression is induced on brain microvascular endothelial cells during *C. ruminantium* infection which, in turn, exerts important influences over the generation of specific $CD4^+$ T cell-mediated immunity. However, it is also possible that the constitutive expression of MHC class I molecules on brain endothelial cells might sustain a $CD8^+$ T cell response.

MHC class II up-regulation on macrophages during bacterial infection, has

been demonstrated to be dependent on bacterial toxin production: the endotoxin LPS by Gram-negative organisms or hemolytic exotoxins by Gram-positive (and some Gram-negative) bacteria (Marshall and Ziegler, 1991). Our data that LPS failed to induce DQα mRNA level, but drastically inhibited its induction by IFN-γ, indicate that *C. ruminantium* infection of BBEC up-regulates MHC class II mRNA via an LPS-independent pathway. Alternatively, the production of a toxin by *C. ruminantium* might play a role in the induction of an anti-bacterial immune response.

Numerous investigators have mentioned the occurence of perivascular leukocyte infiltrates in the brain during *C. ruminantium* infection (Prozesky, 1987). Similar observations have also been reported in other inflammatory diseases affecting the central nervous system, such as multiple sclerosis or its animal model experimental allergic encephalomyelitis (Hickey et al., 1991). In these cases, it has been suggested that the antigen-presenting capacity of brain microvessel endothelial cells, mediated by MHC class II expression and interleukin synthesis, plays a major role in the recruitment, activation and infiltration of cytolytic T lymphocytes into the brain (McCarron et al., 1985; Risau et al., 1990; Fabry et al., 1993). More recently, we observed that MHC class II-restricted interaction between encephalitogenic rat T lymphocytes and syngeneic brain microvessel endothelial cells *in vitro* may actually induce anergy and loss of encephalitogenicity of T cells, these effects being completely or partially prevented, respectively, by IL-1β (S. Bourdoulous, E. Béraud, C. Le Page, A.J. Zamora, A. Ferry, D. Bernard, A.D. Strosberg, P.O. Couraud, submitted for publication). Regarding cowdriosis, our present data suggest that IL-1β, produced by brain endothelial cells early after infection, thus sustains, together with antigen presentation, the activation and proliferation of *C. ruminantium*-specific T cells. The concomitant production of IL-6 could contribute in parallel to the induction of a B cell response, which is in agreement with the observations of a humoral (non-protective) response in infected ruminants (Prozesky, 1987). In addition, the synthesis of IL-8, a chemoattractant for neutrophils (Rampart et al., 1989), might participate in the recruitment of infected neutrophils towards brain endothelium with potential deleterious effects.

Intracellular pathogens often elicit an antigen-non specific response in their host cells. In fact, IFN-γ-activated murine macrophages are known to exert at least part of their bactericidal activity through release of reactive nitrogen intermediates (Stuehr and Marletta, 1985), long before the molecular characterization of cytokine-inducible nitric oxide synthase (Xie et al., 1992). Although we and others have shown that this activity can also be observed in some endothelial cells (Radomski et al., 1990), including rat brain microvessel endothelial cells (Durieu-Trautmann et al., 1993), we constantly failed to detect any nitrite release in the culture supernatant of IFN-γ-stimulated and/or *C. ruminantium*-infected BBEC (not shown). Interestingly, these contrasting results for BBEC and rat brain endothelial cells may reflect a species difference, because human endothelial cells (Woodman et al., 1991)

or macrophages (Cameron et al., 1990) seem to be deficient in IFN-γ-inducible nitric oxide synthase.

In conclusion, our results suggest that brain microvessel endothelial cells, which constitute one of the main cellular targets of *C. ruminantium* during ruminant infection, could directly contribute to the development of an immune response against the pathogen. A detailed analysis of this cellular immunity is currently in progress in our laboratory.

Acknowledgements

We are grateful to Dr V. Lotteau (Institut des Cordeliers, Paris, France), Dr D. Piatier-Tonneau (Institut d'Embryologie, Nogent-sur-Marne, France), and Dr L. Anderson (Uppsala University, Upsalla, Sweden) for the kind gift of cDNA probes. We thank C. Brière for primers synthesis and N. Foignant-Chaverot for expert technical assistance.

This work was supported by grants from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Association pour le Développement de la Recherche sur le Cancer, the Ligue Nationale Française contre le Cancer, the University Paris VII, the Ministère de la Recherche et de l'Enseignement Supérieur, and the European Community CE-DG12 STD3.

References

Zinkernagel, R.M., and P.C. Doherty. 1979. MHC restricted cytotoxic T cell, studies on the biological role of polymorphic major transplantation antigens determining T cell restriction-specificity function and responsiveness. *Adv. Immunol.* 27, 51.

Kaufman, S.H.E. 1988. $CD8^+$ T lymphocytes in intracellular microbial infections. *Immunol. Today* 9, 168.

Gazzinelli, R.T., F.T. Hakim, S. Hieny, G.M. Shearer and A. Sher. 1991. Synergistic role of $CD4^+$ and $CD8^+$ T lymphocytes in IFN-γ production and protective immunity by an attenuated *Toxoplasma gondii* vaccine. *J. Immunol* 146, 286.

Prozesky, L. 1987. The pathology of Heartwater III. A review. *Onderstepoort J. Vet. Res.* 54, 281.

Norval, R.A.I., M.I. Meltzer, and M.J. Burridge. 1992. Distribution, economic importance and control measures for *Cowdria Ruminantium*. In *Recent developments in the control of anaplasmosis, babesiosis and cowdriosis*. T.T. Dolan, ed. ILRAD, Nairobi, Kenya. p. 13.

Van Vliet, A.H.M., F. Jongejan, and B.A.M. Vand Der Zeijst. 1992. Phylogenetic position of Rickettsiae determined by analysis of amplified 16S ribosomal DNA sequences. *Int. J. Syst. Bacteriol.* 42 , 494.

Camus, E. and N. Barré. 1988. Le diagnostic de la cowdriose à partir d'écrasement de cerveau. *Rev. Elev. Méd. Vét. Pays Trop.* 41 ,247.

Jongejan, F. 1991. Protective immunity to Heartwater (*Cowdria Ruminantium* infection) is acquired after vaccination with *in vitro* attenuated *Rickettsiae*. *Infect. Immun.* 59 , 729.

Martinez, D., J.C. Maillard, S. Coisne, C. Sheikboudou and A. Bensaid. 1993. Protection of goats against Heartwater acquired by immunization with inactivated elementary bodies of *Cowdria Ruminantium*. *Vet Immunol. Immunopathol.* (in press).

Jongejan, F. and M.J.C. Thielemans. 1991. Antigenic diversity of *Cowdria Ruminantium* isolates determined by cross-immunity. *Res. Vet. Sci.* 51, 24.

Du Plessis, J.L., P. Berche and L. Van Gas. 1991. T cell-mediated immunity to *Cowdria Ruminantium* in mice, the protective role of,Lyt-2^+ T cells. *Onderstepoort J. Vet. Res.* 58, 171.

Martinez, D., C. Sheikboudou, P.O. Couraud and A. Bensaid. 1993. *In vitro* infection of bovine brain endothelial cells by *Cowdria Ruminantium*. *Res. Vet. Sci.* 55, 258.

Durieu-Trautmann, O., N. Foignant, A. D. Strosberg and P. O. Couraud. 1991. Coexpression of β1- and β2-adrenergic receptors on bovine brain capillary endothelial cells in culture. *J. Neurochem.* 56, 775.

Coutinho, G. C., O. Durieu-Trautmann, A. D. Strosberg and P. O. Couraud. 1991. Catecholamines stimulate the IFN-γ-induced class II MHC expression on bovine brain capillary endothelial cells. *J. Immunol.* 147, 2525.

Bourdoulous, S., O. Durieu-Trautmann, A. D. Strosberg and P. O. Couraud. 1993. Catecholamines stimulate MHC class I, class II and invariant chain gene expression in brain endothelial cells through different mechanisms. *J. Immunol.* 150, 1486.

Piatier-Tonneau, D., P. Turmel, C. Auffray and D. Charron. 1986. Construction of chain- and locus-specific HLA class II DNA probes study of HLA-class II transcripts in leukemias. *J. Immunol.* 137, 2050.

Lotteau, V., L. Teyton, A. Peleraux, T. Nilsson, L. Karlsson, S. L. Schmid, V. Quaranta, and P. A. Peterson. 1990. Intracellular transport of class II MHC molecules directed by invariant chain. *Nature* 348, 600.

Bensaid, A., J. R.Young, A. Kaushal and A. J. Teale. 1991. Pulsed-Field Gel Electrophoresis and its application in the physical analysis of the Bovine Major Histocompatibility Complex. in *Gene mapping techniques and applications.* L.B. Schook, H.A. Lewin, and D.G. McLaren, eds. Marcel Dekker, New York, p 127-158.

Andersson, L. and L. Rask. 1988. Characterization of the MHC class II region in cattle. The number of DQ genes varies between haplotypes. *Immunogenetics* 27, 110.

Van der Poel, J.J., M. Groenen, R. Dijkhof, D. Ruyte and M. Giphart. 1990. The nucleotide sequence of the bovine MHC class II alpha gene DRA, DQA and DYA. *Immunogenetics* 31, 20.

Chomczynski, P. and N. Sacchi. 1987. Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156.

Leong, S.R., G.M. Flaggs, M. Lawman, and P.W. Gray. 1988. The nucleotide sequence for the cDNA of bovine interleukin-1 beta. *Nucl. Ac. Res.* 16, 9054.

Droogmans, L., I. Cludts, Y. Cleuter, R. Kettmann and A. Burny. 1992. Nucleotide sequence of bovine Interleukin-6 cDNA. J. DNA *Sequencing and mapping* 2, 491.

Mukaida, N., M. Shiroo and K. Matsushima. 1989. Genomic structure of the human monocyte-derived neutrophil chemotactic factor IL-8. *J. Immunol.* 143, 1366.

Akli, S., J. Chelly, J. M. Lacorte, L. Poenaru and A. Kahn. 1991. Seven novel tay-sachs mutations detected by chemical mismatch cleavage of PCR-amplified cDNA fragments. *Genomics* 11, 124.

Auffray, C., J.W. lillie, A.J. Korman, J.M. Boss, N. Fréchin, F. Guillemot, J. Cooper, R.C. Mulligan and J.L. Strominger. 1987. Structure and expression of HLA-DQα and -DXα genes, interallelic alternate splicing of the HLA-DQα gene and functional splicing of the HLA-DXα gene using a retroviral vector. *Immunogenetics* 26, 63.

Carbone, F.R., and M.J. Bevan. 1990. Class I-restricted processing and presentation of exogenous cell-associated antigen in vivo. *J. Exp. Med.* 171, 377.

Pfeifer J.D., M.J. Wick, R.L. Roberts, K. Findlay, S.J. Normark and C.V. Harding. 1993. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature* 361, 359.

Nauciel, C. 1990. Role of $CD4^+$ T cells and T-independent mechanisms in acquired resistance to *Salmonella typhimurium* infection. *J. Immunol.* 145, 1265.

Marshall, N.E., and H.K. Ziegler. 1991. Role of bacterial hemolysin production in induction of macrophage Ia expression during infection with *Listeria monocytogenes J. Immunol.* 147, 2324.

Marshall, N.E., and H.K. Ziegler. 1991. Lipopolysaccharide responsiveness is an important factor in the generation of optimal antigen-specific T cell responses during infection with Gram-negative bacteria. *J. Immunol.* 147, 2333.

Hickey, W. F., B. L. Hsu and H. Kimura. 1991. T lymphocytes entry into the Central Nervous System. *J. Neurol. Sci. Res.* 28, 254.

McCarron, R.M., Kempski, O., Spatz, M. and McFarlin, D.E. 1985. Presentation of Myelin Basic Protein by murine cerebral vascular endothelial cells. *J. Immunol.* 134, 3100.

Risau, W., Engelhardt, B. and Wekerle, H. 1990. Immune function of the blood-brain barrier, incomplete presentation of protein (auto-) antigens by rat brain microvascular endothelium *in vitro*. *J. Cell Biol.* 110, 1757.

Fabry, Z., Fitzsimmons, K.M., Herlein, J.A., Moninger, T.O., Dobbs, M.B. and Hart, M.N. 1993. Production of the cytokines interleukin 1 and 6 by murine brain microvessel endothelium and smooth muscle pericytes. *J. Neuroimmunol.* 47, 23.

Rampart, M., J. Van Damme, L. Zonnekeyn and A.G. Herman. 1989. Granulocyte chemotactic protein induces plasma leakage and neutrophil accumulation in rabbit skin. *Am. J. Pathol.* 135, 21.

Stuehr, D.J., and M.A. Marletta. 1985. Mammalian nitrate biosynthesis , mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide *J. Immunol.* 139, 518.

Xie Q.-W., H.J. Cho, J. Calaycay , R.A. Mumford, K.M. Swiderek, T.D. Lee, A. Ding, T. Troso and C. Nathan. 1992. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* 256, 225.

Radomski M.W., R.M.J. Palmer and S. Moncada. 1990. Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci.* USA 87, 10043.

Durieu-Trautmann O, C. Fédérici, C. Créminon, N. Foignant-Chaverot, F. Roux, M. Claire, A.D. Strosberg and P.O. Couraud. 1993. Nitric oxide and endothelin secretion by brain microvessel endothelial cells, regulation by cyclic nucleotides. *J. Cell. Physiol.* 155, 104.

Woodman, J.P., I.H. Dimier and D.T. Bout. 1991. Human endothelial cells are activated by IFN-γ to inhibit *Toxoplasma gondii* replication. *J. Immunol.* 147, 2019.

Cameron, M.L., D.L. Granger, J.B. Weinberg, W.J. Kozumbo, and H.S. Koren. 1990. Human alveolar and peritoneal macrophages mediate fugistasis independently of L-arginine oxidation to nitrite or nitrate. *Am. Rev. Respir. Dis.* 142, 1313.

CHAPTER 5

Recombinant bovine interferon gamma inhibits the growth of *Cowdria ruminantium* but fails to induce major histocompatibility complex class II following infection of endothelial cells

P. Totté [a], N. Vachiery [a], D. Martinez [b], I. Trap [c], K.T. Ballingdall [d], N.D. MacHugh [d], A. Bensaid [c], J. Wérenne [a].

[a] *Université Libre de Bruxelles, Animal Cell Biotechnology, Faculty of Sciences, 50 Av. Franklin Roosevelt, 1050 Bruxelles, Belgium.*

[b] *Centre International en Recherche Agronomique pour le Développement, Département d'Elevage et de Médecine Vétérinaire des Pays Tropicaux (CIRAD-EMVT), BP 1232, 97184 Pointe à Pitre Cedex, Guadeloupe, West Indies.*

[c] *CIRAD-EMVT, 10 rue Pierre Curie, 94704 Maisons-Alfort Cedex, France.*

[c] ILRI, P.O. Box 30709, Nairobi, Kenya.

Veterinary Immunology and Immunopathology, 1996, 53, 61-71.

Abstract

Recombinant bovine IFNγ is a potent inhibitor of *C. ruminantium* growth *in vitro* irrespective of the rickettsial stock, or the origin of the endothelial cells. These results suggest an important role for IFNγ in protective immune responses against *Cowdria ruminantium* infections. Here we also show that IFNγ can induce the expression of MHC class II molecules on the surface of endothelial cells. However, treatment of endothelial cells with IFNγ following infection with *Cowdria* fails to induce MHC class II expression. The implications of this pathogen specific effect on class II expression by endothelial cells with regard to its recognition by the host immune system are discussed.

Introduction

Cowdriosis or heartwater is a tick-borne disease of wild and domestic ruminants caused by the obligate intracellular rickettsia *Cowdria ruminantium* (Cowdry, 1925) that is endemic in sub-saharan Africa and in the West Indies (Uilenberg, 1983). Although a degree of innate resistance against cowdriosis exists among indigenous ruminants, the disease becomes a major problem when exotic breeds are introduced (Provost and Bezuidenhout, 1987). Also, because of the proximity of the West Indies to the American mainland, cowdriosis is a potential threat to the American livestock market (Uilenberg, 1982; Barre et al., 1987).

Animals that survive *Cowdria* infection are immune to homologous challenge but the mechanisms involved in protective immunity against *C. ruminantium* are poorly understood. Preliminary studies suggest that the nature of protective immunity is largely cell-mediated (Uilenberg, 1983). The transfer of Lyt-2^+ T lymphocytes from immunized mice protects susceptible mice against *C. ruminantium* infection (Du Plessis et al., 1991). However, the exact mechanisms of protection effected by immune Lyt-2^+ cells remain to be defined. We have shown recently (Totté et al., 1994) that IFNα, which can be produced by activated T cells (Ho, 1984), is induced in cattle that naturally (without antibiotic treatment) resist an experimental infection with *C. ruminantium*. The growth of the pathogen *in vitro* within bovine vascular endothelial cells (one of the target cells of *C. ruminantium in vivo*) is significantly rertarded by recombinant bovine IFNα, but is not completely blocked (Totté et al., 1994), suggesting that other mechanisms are necessary for the control of the infection. A possible candidate is IFNγ, which is also produced by activated T lymphocytes and has been shown to play an important role in the resistance against several other rickettsias (Byrne and Turco, 1988). Recombinant IFNγ inhibits the *in vitro* growth of *Rickettsia prowazekii* (Turco and Wrinkler, 1983), *Rickettsia tsutsugamushi* Gilliam strain (Hanson, 1991a), *Chlamydia psittaci* (Byrne et al., 1986), *Chlamydia trachomatis* (Shemer and Sarov, 1985) and *Ehrlichia risticii* (Park and Rikihisa, 1991). A protective role for endogenous IFNγ *in vivo* has been demonstrated for *Rickettsia conorii* (Li et al., 1987) and *Chlamydia trachomatis* (Williams et al., 1988; Rank et al., 1992). Another role for IFNγ is in the induction of major histocompatibility complex (MHC) class II expression in a variety of cells including bovine endothelial cells (Coutinho et al., 1991). The primary function of class II MHC molecules is to present foreign antigen-derived peptides to T cells (Hunt et al., 1992) resulting in the initiation of an immune response.

In this report, we show that recombinant bovine IFNγ completely prevents the multiplication of several stocks of *C. ruminantium* in bovine and caprine vascular endothelial cells *in vitro* (part of these results have been published in

the proceedings of the second biennal meeting of the Society of Tropical Veterinary Medicine - STVM 93 - held in Guadeloupe, French West Indies, 2-6 february 1993) (Totté et al., 1993). Furthermore, we describe altered cell surface expression of MHC class II molecules by these cells following infection by *C. ruminantium* and IFNγ treatment.

Materials and methods

Cell cultures

One caprine and two bovine endothelial cell lines were used in this study. Bovine endothelial cells isolated from microvessels of brain cortex (BME) were kindly provided by Dr. G. Tarone (University of Torino, Italy). Bovine endothelial cells from umbilical cord arteries (BUEC) were a kind gift of Dr. F. Jongejan (University of Utrecht, The Netherlands). Caprine endothelial cells (CJE) were isolated from the jugular vein according to established procedures (Schwartz et al, 1991). These cells are positive for factor VIII production as shown by immunofluorescence, (data not shown), indicating that they are of endothelial origin. All these cell lines are fully permissive to *C. ruminantium* growth. All cells were cultured in Dulbecco's modified Eagle medium (Gibco) supplemented with 10 % fetal calf serum (Gibco), penicillin (100 IU ml^{-1}), streptomycin (100 mg ml^{-1}) and L-Glutamine (2mM).

Cowdria stocks and in vitro culture

Three different stocks of *C. ruminantium* were used in this study, the Senegal stock (Jongejan et al., 1988), the Welgevonden stock from South Africa (Du Plessis, 1985) and the Gardel stock from Guadeloupe (Uilenberg et al., 1985). All these stocks were kept as stabilates of *in vitro* infected endothelial cells cryopreserved in sucrose-phosphate-glutamate (SPG) buffer (Bovarnick et al., 1950). Culture of *C. ruminantium* in endothelial cells was carried out as previously described (Totté et al., 1994). Briefly, endothelial cells were grown to confluency in 75 cm^2 tissue culture flasks (Nunc). Cells were infected using SPG-cryopreserved stabilates of *C. ruminantium* diluted in "complete medium" composed of Glasgow minimum essential medium (GMEM, Gibco) supplemented with tryptose phosphate broth (Gibco) at 2.9 g l^{-1}, penicillin (100 IU ml^{-1}), streptomycin (100 mg ml^{-1}); hepes (20 mM, pH 7.0 - 7.2), L-Glutamine (2 mM) and 10 % fetal calf serum (Gibco). The cells were incubated at 37°C in a humidified atmosphere of 5 % CO_2 in air. *C. ruminantium* replicates in intracytoplasmic vacuoles, giving rise to large colonies (morulae) that can be

easily observed under a light microscope (inversed microscope, Leitz Diavert) or after staining of the cells with Giemsa or Diff-quick (Baxter).When more than 70% of the cells were lysed by the rickettsia, the culture supernatant was centrifuged for 15 min at 15 000 g. The pellet obtained from 2 ml of supernatant was resuspended in 1 ml SPG buffer before snap freezing in liquid nitrogen.

One - step growth - yield assay for C. ruminantium

We have studied the effect of recombinant bovine IFNγ on the infectious yield of *C. ruminantium* from endothelial cells infected *in vitro*. This was done by titrating the infectivity of supernatants from endothelial cells infected with *Cowdria* in the presence or absence of IFNγ. Endothelial cells were grown to confluency in 24-well plates (Nunc) prior to infection with *Cowdria* infected supernatant (1 ml per well). Infected superatant was prepared from a culture showing 70-80 % cell lysis due to *C. ruminantium* and diluted two-fold in fresh complete medium prior to use. Triplicates wells were treated with medium alone (control) or with various concentrations of IFNγ at different time intervals. The medium was replaced 24 h after each treatment. In order to confirm the involvement of IFNγ, the experiment was repeated in the presence of neutralising antibodies for bovine IFNγ. The progress of the infection was followed daily by light microscopy. All supernatants were collected 9 days after infection, when control wells (infected but not treated) showed 70-80 % lysis due to the rickettsia. Each supernatant was centrifuged at 15 000 g for 15 min and the pellet resuspended in 100 ml SPG before snap freezing in liquid nitrogen. The infectivity of these supernatants was measured by a TCLD50 assay adapted for *C. ruminantium* (Totté et al., 1994). Results are expressed as % inhibition of *Cowdria* yield (± standard deviation) compared to the control.

IFN, IFN assay, and anti-IFN antibodies

Recombinant bovine interferon gamma (rBoIFNγ, specific activity $2x10^6$ U mg^{-1}) produced in *E. Coli* and neutralizing antibodies to BoIFNγ were kindly donated by Dr. R. Steiger (CIBA - GEIGY). The titer of rBoIFNγ was regularly measured by the classical test of reduction of the cytopathogenicity of vesicular stomatitis virus (Stewart, 1979) on Madin Darby bovine kidney cells (MDBK). The antiviral activity was expressed in laboratory units; no international reference standards are available for bovine IFNs. One unit of antiviral activity is defined as the reciprocal of the dilution that gives 50% protection against viral challenge under standard conditions. In our test, a 10 fold dilution of anti-

BoIFNγ antibodies was shown to neutralize 1.10^3 U of rBoIFNγ.

Flow cytometry analysis of endothelial cell surface class II molecules

BUE and CJE cells were grown and infected with the Gardel stock of *C. ruminantium* as described above. A total of 5 U ml^{-1} of recombinant IFNγ were added to each culture including non-infected samples at 4 and 24 h after infection. After 72 h, endothelial cell layers were gently trypsinized and washed in RPMI medium containing 10 % fetal calf serum. Each sample was resuspended in 100 ml of FACS medium (RPMI 1640, 2% normal horse serum and 0.02% sodium azide) and divided in two aliquots where a control mAb (VC13) or an anti MHC class II mAb (VC9) were added at a final dilution of 1/1000. After 45 min incubation, the cells were washed three times and resuspended in 50 ml of FACS medium containing FITC labelled goat anti-mouse Ig (Caltag, San Francisco, CA) diluted 1/100. The cells were further incubated for 45 min and washed three times before analysis on a fluorescence activated cell sorter (FACSort apparatus, Becton Dickinson, San Jose, CA). Monoclonal antibody VC13 specifically recognizes BoCD1 molecules (MacHugh, personal communication). The specificity of mab VC9 for MHC class II molecules was determined by tissue distribution and immunoprecipitation studies and by its reactivity to previously described L-cell transfectants (Ballingall et al, 1995) as shown in Fig.1.

Results

The effect of rBoIFNγ on the yield of *C. ruminantium* infectious particles from vascular endothelial cells was studied *in vitro*. rBoIFNγ reduced the yield of infectious *C. ruminantium* (Senegal stock) from BME cells in a dose-dependent manner (Fig. 2). Although pretreatment of BME cells with rBoIFNγ 24 h. prior to infection resulted in significant inhibition of *Cowdria* growth, rBoIFNγ was more effective when added after adsorption of *C. ruminantium* (e.g., 24 h. after infection; Fig. 2). Under these conditions, 100 % inhibition of *C. ruminantium* yield was achieved with as litle as 0.5 U/ml of rBoIFNγ (Fig. 2). The inhibitory effect was completely reversed by addition of rBoIFNγ-specific antibodies to the medium (Fig.2). Inhibition was unaffected by the addition of 100 U ml^{-1} of polymyxin B (a known chelator of bacterial endotoxins; not shown). Although 100 % reduction of the *Cowdria* infectious yield was observed 9 days post-infection, when cells were treated with up to 5 U ml^{-1} of rBoIFNγ, colonies of *C. ruminantium* were still visible in the cytoplasm of some cells. Therefore the progression of the infection was delayed by rBoIFNγ but not completely

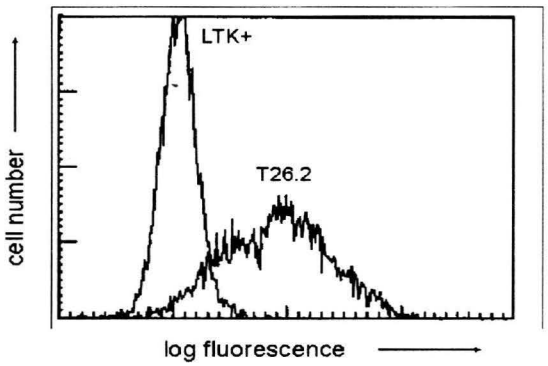


Fig. 1. The MHC class II specificity of the mAb VC9 was determined by FACS analysis of MHC class II expressing L-cell transfectant T26.2 and control thymidine kinase transfected line LTK+. Cells were labelled with the mAb VC9 followed by FITC-IgG antimouse Ig as a second stage.

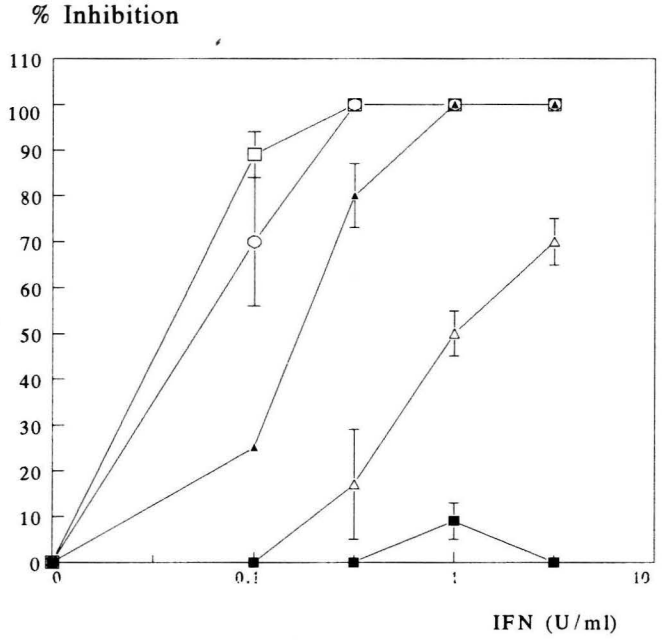


Fig. 2. Inhibition by rBoIFNγ of *C. ruminantium* (Senegal) infectious yield from BME cells. rBoIFNγ was added to the medium at different concentrations and time intervals: 24 h. before infection (Δ); at day 0 (▲); at day 1(o) and at days 0 and 1 (□). Some wells received rBoIFNγ at days 0 and 1 together with anti-rBoIFNγ (■). Data are mean values (± standard deviation) of four experiments.

prevented. However, when the cells were treated with 10 U ml^{-1} of rBoIFNγ at days 0 and 1, colonies of *C. ruminantium* were not observed for up to 30 days post-infection (not shown). Cytotoxicity of rBoIFNγ for uninfected or *C. ruminantium* infected BME and BUE cells was observed only when 50 U ml^{-1} or more were added to the medium for three consecutive days.

As shown in Fig. 3, bovine endothelial cells are sensitive to the anti-*cowdria* effect of rBoIFNγ regardless their tissue of origin. Indeed, microvasculature (BME) or macrovasculature (BUE) endothelial cells became equally non-permissive to *C. ruminantium* replication after rBoIFNγ treatment (Fig. 3). Caprine endothelial cells from jugular veins (CJE) were also sensitive to the anti-*cowdria* effect of rBoIFNγ (Fig. 3).

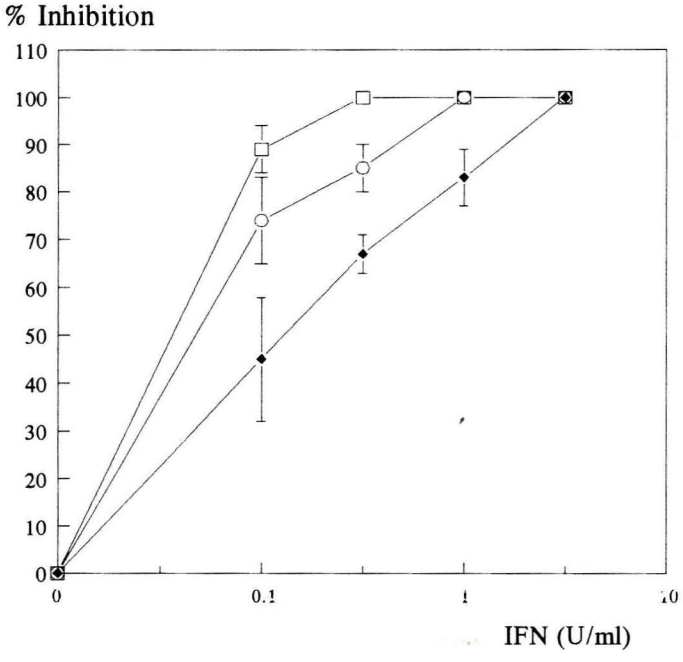


Fig.3. rBoIFNγ mediated inhibition of *C. ruminantium* (Senegal) infectious yield in different types of vascular endothelial cells. BME (□), BUE (o) and CJE (◆) cells were treated with various concentrations of rBoIFNγ at day 0 and 1 post-infection. Data are mean values (± standard deviation) of two experiments.

The growth of three stocks of *C. ruminantium* originating from different geographical locations were found to be efficiently inhibited by rBoIFNγ in BUE

cells (Fig. 4). Although the *C. ruminantium* stocks used have been shown previously to be antigenically different in cross-immunity trials (Jongejan et al., 1988), our results demonstrate a dose-dependent inhibition of *Cowdria* growth *in vitro* irrespective of the stock used.

The endothelial cells used in this study, whether infected with *Cowdria* or not, did not constitutively express cell surface MHC class II molecules (Fig. 5a, 5b). Treatment with 5 U ml^{-1} of rBoIFNγ induced MHC class II expression on both BUE and CJE cells. Indeed, 55% and 46% of treated BUE and CJE cells respectively were positive for the mAb VC9 (Fig. 5c). In contrast, a significant inhibition of the expression of MHC class II molecules was observed on the surface of cells treated with rBoIFNγ with only 10 % and 7 % of BUE and CJE

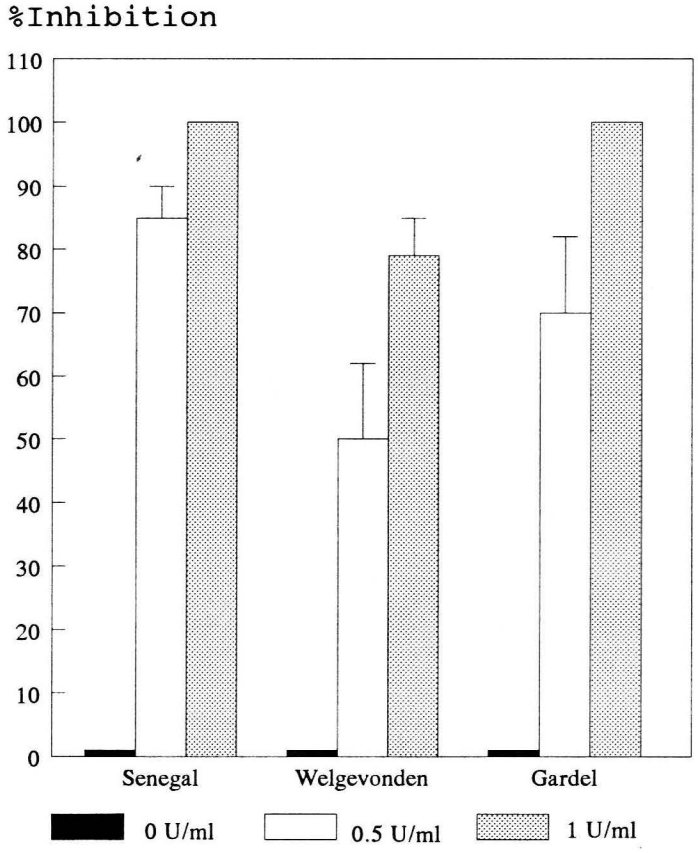


Fig.4. Susceptibility of three different strains of *C. ruminantium* to the inhibitory effect of rBoIFNγ. BUE cells were treated with rBoIFNγ at day 0 and 1 post-infection. Data are expressed as mean values (± standard deviation) of triplicates.

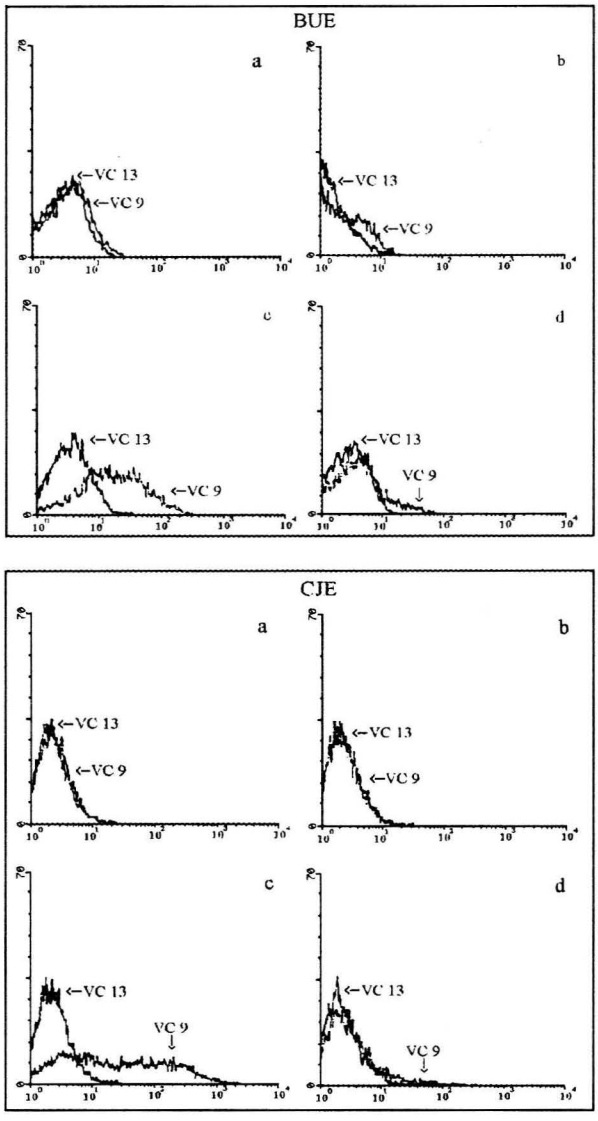


Fig.5. FACS analysis of BUE and CJE cells. Cells were labelled with an anti MHC class II mAb (VC9) or a mAb control (VC13) as described in material and methods. The Y axis represents the number of cells while the X axis correspond to the intensity of fluorescence; (a) non-infected, (b) *C. ruminantium* infected, (c) non-infected and treated with rBoIFNγ and (d) infected and treated with rBoIFNγ.

cells, respectively, expressing the antigens after infection with *C. ruminantium* (Fig. 5d).

Discussion

We have established that rBoIFNγ is a potent inhibitor of the growth of *C. ruminantium* in vascular endothelial cells *in vitro*. Growth of *C. ruminantium* in these cells is significantly inhibited at very low concentrations of rBoIFNγ, compared to that shown for other rickettsia in other cells. In our model, 10 U ml^{-1} of rBoIFNγ is capable of preventing the formation of *C. ruminantium* colonies, whereas 100 U ml^{-1} of human recombinant IFNγ are required to prevent infection of human epithelial cells by *Chlamydia trachomatis* (Shemer and Sarov, 1985). This may reflect differences between species and the type of cells used. We have previously demonstrated that bovine endothelial cells are 10 to 20-fold more sensitive to the antiviral activity of bovine IFNs compared to bovine epithelial cells (Totté et al., 1993).

It is also apparent from this study that rBoIFNγ acts on the host cells to render them refractory to *C. ruminantium* replication rather than directly on the extracellular organism. Indeed, treatment of the cells with rBoIFNγ at a time when extracellular *Cowdria* has been removed is more efficient than when simultaneous treatment and infection is performed. The formation of *C. ruminantium* colonies is prevented in vascular endothelial cells treated with rBoIFNγ, but the exact stage of the developmental cycle of *C. ruminantium* on which rBoIFNγ acts is not known. The inhibitory effect of rBoIFNγ on the infectious yield of *C. ruminantium* cannot be explained by specific lysis of infected cells (as it was shown for *Chlamydia trachomatis* (Byrne et al., 1988), *Rickettsia prowazekii*, (Turco and Wrinkler, 1983), and *Rickettsia tsutsugamushi* (Hanson, 1991a)) since no cytotoxic effect was detected at concentrations of rBoIFNγ that prevented the formation of *C. ruminantium* colonies in endothelial cells. Involvement of oxygen catabolism metabolites, tryptophan degradation, nitric oxide and transferrin receptors in the anti-*cowdria* effect of rBoIFNγ in vascular endothelial cells is currently under investigation in our laboratory.

The inhibitory effect of rBoIFNγ has been shown to vary between different strains of *Rickettsia tsutsugamushi*, with certain strains being unaffected by IFNγ (Hanson, 1991b). Here, we show that three strains of *C. ruminantium* from different parts of the world (Senegal, South Africa and Guadeloupe), with different antigenic properties are each susceptible to rBoIFNγ mediated inhibition *in vitro*. This inhibitory effect is independent of the tissue of origin of the endothelial cell lines. Caprine endothelial cells are also equally

susceptible to the inhibitory effects of rBoIFNγ.

Thus, rBoIFNγ appears to be a very powerful inhibitor of *C. ruminantium* growth *in vitro*, acting independently of the rickettsial stock, endothelial cell origin and ruminant species. These results suggest a direct role for rBoIFNγ in protective immune responses against *C. ruminantium* infections.

Endothelial cells have the capacity to process and present antigens to T cells in an MHC class II restricted manner (Sedwick et al., 1990). However our results suggest that this is not the case during *Cowdria* infections since infected endothelial cells do not express MHC class II molecules and have a markedly reduced capacity to do so even following rBoIFNγ treatment. These results suggest that a direct interaction (leading to cytotoxicity or cytokine production) between $CD4^+$ T cells and *Cowdria* infected endothelial cells is unlikely *in vivo*. The involvement of MHC class I restricted $CD8^+$ T lymphocytes and $CD4^+$ helper cells activated by more conventional pathways (e.g., macrophage) in the immunology and immunopathology of cpwdriosis is more likely to occur.

Although endothelial cells infected with *Cowdria* are highly sensitive to treatment with IFNγ resulting in a significant reduction in *Cowdria* growth, treatment fails to induce cell surface MHC class II expression. How a few remaining colonies can influence class II induction by IFNγ is not yet understood. IFNγ may act on the later stage of the *Cowdria* growth cycle while inhibitory factors are produced by earlier stages.

Our results suggest that *C. ruminantium* has developed mechanisms to escape recognition by the host immune response. These include the production of inhibitory factors that greatly reduce the capacity of endothelial cells to express MHC class II molecules. As *C. ruminantium* is extremely sensitive to IFNγ it is likely to have developed means to limit the production of this cytokine. One of these may be to prevent MHC class II restricted activation of Th1 subsets of T lymphocytes that produce IFNγ. Further experiments to study the effect of *Cowdria* infection on MHC class I expression and the constitutive expression of MHC class II molecules by professional antigen presenting cells (monocytes, dendritic cells) are planned.

Acknowledgements

The work presented here was supported in part by the European Community (DG XII) under contract TS3-0115-C, entitled: "Integrated control of Dermatophilosis and Cowdriosis of livestock".

References

1. Ballingall, K.T., Dutia, B.M., Hopkins, J. and Wright, H., 1995. Analysis of the fine specificities of sheep major histocompatibility complex class II-specific monoclonal antibodies using mouse L-cell transfectants. *Animal Genetics*, 26, 79-84.

2. Barre, N., Uilenberg, G., Morel, P.C. and Camus, E., 1987. Danger of introducing heartwater onto the american mainland, Potential role of indigenous and exotic *Amblyomma* ticks. *Onderstepoort J. of Vet. Res.* 54, 405-417.

3. Bovarnick, M.R., Miller, J.C. and Snyder, J.C., 1950. The influence of certain salts, amino acids, sugars and proteins on the stability of rickettsiae. *J. Bacteriol.* 59, 509-522.

4. Byrne, G.I. and Turco, J., 1988. Interferon and nonviral pathogens. In, G.I. Byrne and J. Turco (Editors), *Immunology series*, vol. 42, 317 p.

5. Byrne, G.J., Lehmann, L.K. and Landry, G.J., 1986. Induction of tryptophan catabolism is the mechanism for gamma-interferon-mediated inhibition of intracellular *Chlamydia psittaci* replication in T24 cells. *Infect. Immun.* 53, 347-351.

6. Byrne, G.I., Grubbs, B., Marshall, T.J., Slachter, J. and Williams, D.M., 1988. Gamma IFN-mediated cytotoxicity related to murine *Chlamydia trachomatis* infection. *Infect. Immun.* 56, 2023-2027.

7. Coutinho, G.C., Durieu-Trautmann, O., Strosberg, A.D. Couraud, P.O., 1991. Catecholamines stimulate the IFN gamma induced class II MHC expression on bovine brain cappillary endothelial cells. *J. Immunol.* 147, 2525-2529.

8. Cowdry, E.V., 1925. Studies on the etiology of heartwater. I. Observations of a rickettsia, *Rickettsia ruminantium* (n.sp.), in the tissues of infected animals. *J. Exp. Med.* 42, 231-252.

9. Du Plessis, J.L., 1985. A method for determining the *Cowdria ruminantium* infection rate of *Amblyomma hebraeum*, effects in mice infected with tick homogenates. *Onderstepoort J. Vet. Res.* 52, 55-61.

10. Du Plessis, J.L., Berche, P. and Van Gas, L., 1991. T cell-mediated immunity to *Cowdria ruminantium* in mice, the protective role of LYT-2^+ cells. *Onderstepoort J. Vet. Res.* 58, 171-179.

11. Hanson, B., 1991a. Susceptibility of *Rickettsia tsutsugamushi* Gilliam to Gamma interferon in cultured mouse cells. *Infect. Immun.* 59, 4125-4133.

12. Hanson, B., 1991b. Comparative susceptibility to mouse Interferons of *Rickettsia tsutsugamushi* strains with different virulence in mice and of *Rickettsia rickettsii*. *Infect. Immun.* 59 , 4134-4141.

13. Ho, M., 1984. Induction and inducers of IFN. Interferon, volume I, General and applied aspects. Elsevier, Amsterdam, 79-124.

14. Hunt, D.F., Michel, H.,Dickinson, T.A., Shabanowitz, J., Cox, A.L., Sakagushi, K., Appella, E., Grey, H.W. and Sette, A., 1992. Peptides presented to the immune system by the murine class II MHC molecule I-A^d. *Science* 256, 1817-1820.

15. Jongejan, F., Uilenberg, G., Franssen, F.F.J., Gueye, A. and Nieuwenhuis, J., 1988. Antigenic differences between stocks of *Cowdria ruminantium*. *Res. Vet. Sci.* 44, 186-189.

16. Jongejan, F., Vogel, S.W., Gueye, A. and Uilenberg, G., 1993. Vaccination against heartwater using *in vitro* attenuated *Cowdria ruminantium* organisms. *Rev. Elev. Méd. Vét. Pays Trop.* 46, 223-227.

17. Li, H., Jerrells, T.R., Spitalny, G.L. and Walker, D.H., 1987. Gamma interferon as a crucial host defense against *Rickettsia conorii in vivo*. *Infect. Immun.* 55, 1252-1255.

18. Park, J. and Rikihisa, Y., 1991. Inhibition of *Ehrlichia risticii* infection in murine peritoneal macrophages by gamma interferon, a calcium ionophore, and concanavalin A. *Infect. Immun.* 59, 3418-3423.

19. Provost, A. and Bezuidenhout, J.D., 1987. The historical background and global importance of heartwater. *Onderstepoort J. Vet. Res.* 54 , 165-169.

20. Rank, R.G., Ramsey, K.H., Pack, E.A. and Williams, D.M., 1992. Effect of gamma interferon on resolution of murine Chlamydial genital infection. *Infect. Immun.* 60, 4427-4429.

21. Schwartz, B., Vicart, P., Delouis, C. and Paulin, D., 1991. Mammalian cell lines can be efficiently established *in vitro* upon expression of the SV40 large T antigen driven by a promoter sequence derived from the human vimentin gene. *Biol. Cell*, 73, 7-14.

22. Sedwick, J.D., Hughes, C.C., Males, D.K., MacPhee, I.A.M. and Meulen, V.T., 1990. Antigen-specific damage to brain vascular endothelial cells mediated by encephalitogenic $CD4^+$ T cell lines *in vitro*. *J. Immunol.* 145, 2474-2481.

23. Shemer, Y. and Sarov, I., 1985. Inhibition of growth of *Chlamydia trachomatis* by human gamma Interferon. *Infect. Immun.* 48, 592-596.

24. Shemer, Y. and Sarov, I., 1988. Interferon and nonviral pathogens. In, G.I. Byrne and J. Turco (Editors), *Immunology series*, vol. 42, 73-94.

25. Stewart, W.E. II., 1979. The Interferon System. Vienna. Springer-Verlag, New York.

26. Totté, Ph., Blankaert, D., Zilimwabagabo, P. and Werenne, J., 1993. Inhibition of *Cowdria ruminantium* infectious yield by interferons alpha and gamma. *Rev. Elev. Med. Vet. Pays Trop.* 46, 189-194.

27. Totté, Ph., Jongejan, F., de Gee, A.L.W. and Werenne, J., 1994. Production of interferon alpha in *Cowdria ruminantium* infected cattle and its effect on infected endothelial cell cultures. *Infect. Immun.* 62, 2600-2604.

28. Turco, J. and Wrinkler, H.H., 1983. Cloned mouse interferon gamma inhibits the growth of *Rickettsia prowazekii* in cultured mouse fibroblasts. *J. Exp. Med.* 158, 2159-2164.

29. Turco, J., and Wrinkler, H.H., 1984. Effect of mouse lymphokines and cloned mouse interferon gamma on the interaction of *Rickettsia prowazekii* with mouse macrophage-like RAW264,7 cells. *Infect. Immun.* 45, 303-308.

30. Uilenberg, G., 1982. Experimental transmission of the Gulf coast tick *Amblyomma maculatum* , danger of introducing heartwater and benign african theileriasis onto the american mainland. *Am. J. Vet. Res.* 43, 1279-1282.

31. Uilenberg, G., 1983. Heartwater (*Cowdria ruminantium* infection), current status. *Adv. Vet. Sci. and Comp. Med.* 27, 427-480.

32. Uilenberg, G., Camus, E. and BarrÈ, N., 1985. Quelques observations sur une souche de *Cowdria ruminantium* isolée en Guadeloupe (Antilles françaises). *Rev. Elev. Med. vet. Pays trop.* 38, 34-42.

33. Williams, D.M., Byrne, G.I., Grubbs, B., Marshall, T.J. and Schachter, J.S., 1988. A role *in vivo* for gamma interferon in control of pneumonia caused by *Chlamydia trachomatis* in mice. *Infect. Immun.* 56, 3004-3006.

CHAPTER 6

Comparative efficacy of Freund's and Montanide ISA50 adjuvants for the immunisation of goats against hearwater with inactivated *Cowdria ruminantium*

D. Martinez[a], J.M. Perez[a], C. Sheikboudou[a], A. Debus[a], A. Bensaid[b].

[a] *Centre International en Recherche Agronomique pour le Développement, Département d'Elevage et de Médecine Vétérinaire des Pays Tropicaux (CIRAD-EMVT), BP 1232, 97184 Pointe à Pitre Cedex, Guadeloupe, West Indies.*

[b] *CIRAD-EMVT, BP 5035, 34032 Montpellier, France.*

Veterinary Parasitology, 1996, 67 (3-4), 175-184.

Abstract

Two vaccines, based on inactivated elementary bodies of *Cowdria ruminantium*, one formulated in Montanide ISA 50, the other in Freund's adjuvant, were compared in goats. Administered twice subcutaneously with an interval of 81 days, both protected 3 out of 5 goats against a very severe challenge, lethal for all 14 control goats, 3.5 months after the second injection. Both vaccines elicited similar antibody levels.

The protection afforded by the Montanide ISA 50 vaccine was tested 15 and 17 months after the second injection of the vaccine. Three out of 6 and 5 out of 6 goats, respectively, survived a challenge which killed all 4 control goats used on each occasion. Antibodies were still detectable in the immunised goats. The level of protection appears to be influenced by the dose of virulent *C. ruminantium* used for the challenge.

As any stock of *C. ruminantium* can be incorporated in order to cover the antigenic repertoire of the organism, this kind of inactivated vaccine can now be tested in the field.

Introduction

The tick-borne rickettsia *Cowdria ruminantium* is the causative agent of heartwater, an infectious disease of ruminants in sub-Saharan Africa (Provost and Bezuidenhout, 1987) and in the Caribbean islands (Perreau et al., 1980; Uilenberg et al., 1984) from where it threatens the American mainland (Barré et al., 1987). On a global scale, it is considered to be one of the economically most important tick-borne disease of ruminants, with babesiosis, anaplasmosis and theileriosis (Uilenberg, 1995). Protection of livestock is achieved by controlling *Amblyomma* ticks (Jongejan and Uilenberg, 1994) and by immunisation of animals using an infection and treatment method (Bezuidenhout, 1989). Despite the fact that it induces a prolonged and solid protection, this method of vaccination is far from ideal. A great deal of research effort has therefore been directed toward the development of safer vaccines since the first description of an *in vitro* cultivation system for *Cowdria* (Bezuidenhout et al., 1985). The attenuation of the Senegal stock of the parasite has been obtained and has been shown to confer protection against an homologous challenge (Jongejan, 1991). However this isolate gives a limited cross-protection against other stocks of Cowdria (Jongejan et al., 1993) and attempts at attenuating other stocks have failed. Récently, protection of goats against heartwater was obtained by immunisation with inactivated elementary bodies of *Cowdria* formulated in Freund's adjuvant (Martinez et al., 1994) and the results were confirmed in sheep (Mahan et al., 1995). However, Freund's adjuvant has side effects which make it unsuitable for commercial use even in veterinary medicine (Edelman, 1980). The introduction of highly purified mineral oils of low viscosity and new injectable emulsifying agents has resulted in the reduction of side effects and in the development of many oil based veterinary vaccines. Amongst the variety of adjuvants available, Montanide ISA 50 has proved to be very effective in immunisation against various microorganisms causing animal or human diseases (Castrucci et al., 1989; Cook et al., 1990; Jones et al. 1990; Roy et al., 1992; Ganne et al., 1994).
In this study, we compared the efficiency of Freund's adjuvant and Montanide ISA 50 in inducing a protective immune response against cowdriosis in goats.

Materials and methods

Preparation of Cowdria antigens

Cowdria ruminantium organisms (Gardel stock, Uilenberg et al., 1985) were produced in caprine endothelial cells by conventional methods (Martinez et al., 1990). Antigens for immunisation were produced as described previouly (Martinez et al., 1994). Briefly, supernatants of lysing cultures were harvested and centrifuged at 1500 rpm for 15 min to pellet host cell debris. The supernatant was saved and the

pellet resuspended in 0.1M PBS, pH 7.2, shaken vigourously and centrifuged again at 1500 rpm for 15 min. The procedure was repeated 3 times. The supernatants cleared from cell debris were pooled and then centrifuged at 30 000 g for 30 min at 4°C to pellet the organisms. The pellet containing elementary bodies of *Cowdria* was resuspended in PBS and the protein content determined by the method of Bradford (1976). After verification of its purity by staining with eosine-methylene blue (RAL 555), the antigen was inactivated overnight at 4°C with 0.1% sodium azide. The inactivation of *Cowdria* was verified by inoculating monolayers of caprine endothelial cells which were checked during 4 weeks for the absence of cell lysis and then stained and examined microscopically. Antigens were stored at -20°C until used.

Titration of Cowdria inoculum

Virulent preparations consisting in supernatant of cultures showing approximately 80% of lysis were titrated in tissue culture microplates as described by Totté et al. (1993) with some modifications. One hundred µl of two fold dilutions of the infected supernatant were inoculated in each well of 96 wells microplates coated with monolayers of caprine endothelial cells. Eight replicates per dilution were done. The plates were incubated at 37°C with 5% CO2 in a humid atmosphere and checked regularly for the appearence of a cytopathogenic effect (CPE). Half of the medium was changed after one week and the plates were read at 10 and 15 days after inoculation. The proportion of wells showing a CPE was recorded for each dilution of the inoculum. The titre giving a CPE in 50% of the wells was calculated by the method of Reed and Muench (1938) and expressed in TCLD50 (tissue culture lethal dose 50%).

Adjuvants and vaccine formulation

The efficacy of Freund's adjuvant used in previous experiments (Martinez et al., 1994) was compared with that of the oil adjuvant Montanide ISA50 (SEPPIC). Water in oil (w/o) emulsion vaccines were formulated prior to use by 10 passages through a 23 gauge needle. Both adjuvants were emulsified with the *Cowdria* antigen diluted in PBS (400 µg and 600 µg of protein per ml for the first and the booster injections, respectively) in a 50:50 ratio. Formulations of PBS with each adjuvant without *Cowdria* antigen were also prepared in the same manner.

Table 1
Results of the challenge of goats vaccinated with inactivated *C. ruminantium* formulated in Freund's adjuvant (FA) or in ISA50 (ISA). Ag: *Cowdria* antigen

	Group 1 [a]					Group 2 [b]		Group 3 [c]	
	Ag + ISA	PBS + ISA	Ag + FA	PBS + FA	Naive	Ag + ISA	Naive	Ag + ISA	Naive
Number of goats	5	5	5	5	5	6	4	6	4
Incubation period (days)									
Mean	6	6	5	6	6	6.5	4.5	8	11
Min.–max.	4–7	5–7	4–7	3–7	5–7	5–9	3–10	8	11
Max. temperature (°C)									
Mean	41.2	40.8	41.6	41.1	40.8	41.7	40.8	41.7	41.3
Min.–max.	40.9–41.5	40.0–41.2	41.3–41.9	40.9–41.4	40.0–41.4	40.1–42.3	40.0–41.4	41.5–41.8	40.2–41.6
Duration of hyperthermia (days)									
Mean	6	3	7	5	2.5	5	7.5	7	2.5
Min.–max.	5–7	1–4	4–9	3–8	1–4	1–7	5–9	5–8	1–4
Survival ratio	3/5	0/5	3/5	0/4 [d]	0/5	3/6	0/4	5/6	0/4
Time to death (days)									
Mean	11	8	14.5	10	8.5	11.5	10.5	14	13.5
Min.–max.	11	8	14–15	9–11	8–9	10–13	10–12	14	12–15

[a] Time to challenge: 3.5 months. Challenge dose: 2 ml of untitrated supernatant.
[b] Time to challenge: 15 months. Challenge dose: 50 TCLD50.
[c] Time to challenge: 17 months. Challenge dose: 10 TCLD50.
[d] One out of the five goats of this group died accidentally before the challenge.

Experimental animals

Forty-five adult goats were purchased from Les Saintes Islands (French West Indies), free of heartwater and *Amblyomma* ticks. This population of goats proved to be highly susceptible to cowdriosis since a 100% mortality of the animals injected with the Gardel strain is observed in absence of treatment. The animals were aged 1 to 3 years and weighed between 10 and 26 kg. They were maintained on tick-proof duck-boards and fed appropriate concentrate (average of 400 g/day/animal) with water and hay *ad libitum*. The animals were allowed to adapt to their new environmental conditions for 1 month before starting the experiment. After immunising or challenge inoculations, rectal temperature and symptoms were recorded daily between 8 and 9 a.m. by the same animal keeper.

Immunisation and challenge procedure

Groups of goats were defined as described in Table 1. Animals were assigned to their group according to their sex and weight to make the groups as homogeneous as possible. All animals were vaccinated twice subcutaneously at 81 days interval with 2 ml of the appropriate vaccinal preparation : 17 goats with *Cowdria* antigen in ISA50 (400 µg and 600 µg of protein per animal for the first and the booster injection, respectively), 5 control goats with PBS in ISA50, 5 goats with *Cowdria* antigen in Freund's adjuvant (first injection with 400 µg of antigen in Freund's complete adjuvant FCA, booster injection with 600 µg in incomplete Freund's adjuvant IFA), 5 goats with PBS in FCA and IFA as before. Thirteen goats remained as unvaccinated controls.

Animals were challenged 3.5 months (group 1), 15 months (group 2) and 17 months (group 3) after boosting by intravenous injection of a virulent culture supernatant as described in Table 1.

Serology

Sera were collected before and at multiple time points during the trial for assay of specific antibody to *C. ruminantium* by an ELISA method (Martinez et al., 1993).

Results

Protection against a lethal challenge

Table 1 summarizes the results of virulent challenges performed 3.5 months , 15 months and 17 months after boosting. After each challenge, all naive or adjuvant

control animals died of heartwater as confirmed by the presence of *C. ruminantium* colonies in the brain capillaries.

Challenge 1 was done by intravenous injection of 2 ml of untitrated virulent culture supernatant per goat in order to compare the results with those of previous experiments performed in similar experimental conditions (Martinez et al., 1994). After this challenge, three out of five goats vaccinated with preparations containing *Cowdria* antigen survived whether the formulation of the vaccine was done with Freund's adjuvant (FA) or ISA50 adjuvant. The heavy challenge dose used resulted in a short incubation period ranging between 3 and 7 days depending on the animal. The mean incubation period was not different between groups.

At challenge 2, three out of six goats vaccinated with *Cowdria* in ISA50, survived the inoculation of 50 TCLD50 of virulent *Cowdria*. For most of the animals the incubation period was still short, ranging between 3 and 10 days. The mean incubation period was 5.5 days for challenge 1 and 2.

For challenge 3, a lower dose of 10 TCLD50 per goat was therefore used to try to reproduce a normal incubation period of around 12 days. This dose proved to be close to that of a natural challenge by ticks since it resulted in an incubation period of 11 days in all naive control animals. Vaccinated animals had a shorter incubation period of 8 days and five out of six survived this challenge.

Whatever the vaccination procedure and the outcome of the challenge, all animals developed a high hyperthermia with a peak which was not significantly different between groups. The febrile period lasted 5 to 7 days on the average in groups vaccinated with antigen and it was not dose dependant. Only one goat showed a febrile reaction of one day. At the beginning of the febrile period, the goats did not show any symptoms except hyperthermia. After 2 to 4 days, the animals showed a loss of appetite, a dull hair-coat, and listlessness. They recovered their normal behaviour quickly after their rectal temperature reached a normal value. The decline of the temperature was not always the sign of healing since seven out of eight vaccinated goats died between 1 and 4 days after their temperature started to decrease and reached a subnormal value. Even in control groups, ten out of 13 goats died 1 (8 goats) to 3 days (2 goats) after the temperature began to decrease.

Although the number of goats was low, the time to death was shorter in control than in vaccinated groups at challenge 1. This difference was not apparent with the lower doses of *Cowdria* used at challenge 2 and 3.

Adverse reactions to the vaccination

Groups of goats injected with adjuvants developed a slight hyperthermia for a period of several days with rectal temperatures 0.5 to 1°C above that of the controls. After the priming injection, hyperthermia lasted from day 9 to 14 when FA was used with PBS or *Cowdria* antigen, from day 9 to 11 for PBS in ISA50, and from day 6 to 11 for antigen in ISA50. This effect was more pronounced in groups inoculated with

adjuvant plus antigen than in groups inoculated with adjuvant alone. At boosting, PBS in ISA50 did not provoke any hyperthermia whereas PBS in FA induced a 0.5°C increase in rectal temperature for 8 days after the injection. An hyperthermia of 1°C was observed in animals vaccinated with antigen in FA and antigen in ISA50 from day 2 to7 and day 3 to 8 respectively.

All animals developed a sterile abscess at the site of injection which resorbed spontaneously only after several weeks.

Serology

All goats were seronegative before vaccination. Animals injected with vaccinal preparations containing antigen developed antibodies to *Cowdria* whereas animals in naive or adjuvant control groups remained seronegative all along the experiment. Goats vaccinated with antigen in FA or ISA50 developed similar levels of antibody but antibodies were detectable earlier when using ISA50 (Fig. 1a). Antibodies remained at a detectable level all along the experiment and their titre increased 2 (challenge 1 and 2) to 3 fold (challenge 3) after the virulent injections (Fig. 1b, 1c, 1d).

Discussion

Results presented here confirm a first report demonstrating the possibility to induce a homologous immunity against cowdriosis in susceptible goats by vaccination with an inactivated preparation of *C. ruminantium* elementary bodies formulated in FA. The level of protection (60%) obtained in the present study with FA is in agreement with that obtained in previous vaccination trials (50 and 80%) conducted in similar experimental conditions (Martinez et al., 1994). In Zimbabwe, Mahan et al. (1995) were able to protect 5 out of 5 sheep by vaccination with inactivated *Cowdria* in FA. A lower susceptibility of their sheep population compared to creole goat or the use of a less pathogenic stock of Cowdria may have been responsible for this better percentage of protection since 3 of their 5 control sheep survived the challenge. One of the control sheep even did not show any hyperthermia or clinical sign of cowdriosis.

Despite its high adjuvant activity, FA has inflammatory side-effects which make it inappropriate for field use. For this reason, its efficacy was compared to that of Montanide ISA50, a licensed adjuvant based on mannide mono-oleate emulsifying agent in mineral oil. The number of animals surviving a virulent challenge administered 3.5 months after boosting was equal using these 2 immunoenhancers. These results are consistent with other studies reporting that ISA50 used in different vaccinal preparations provides a very good immunopotentiation (Cook et al., 1990)

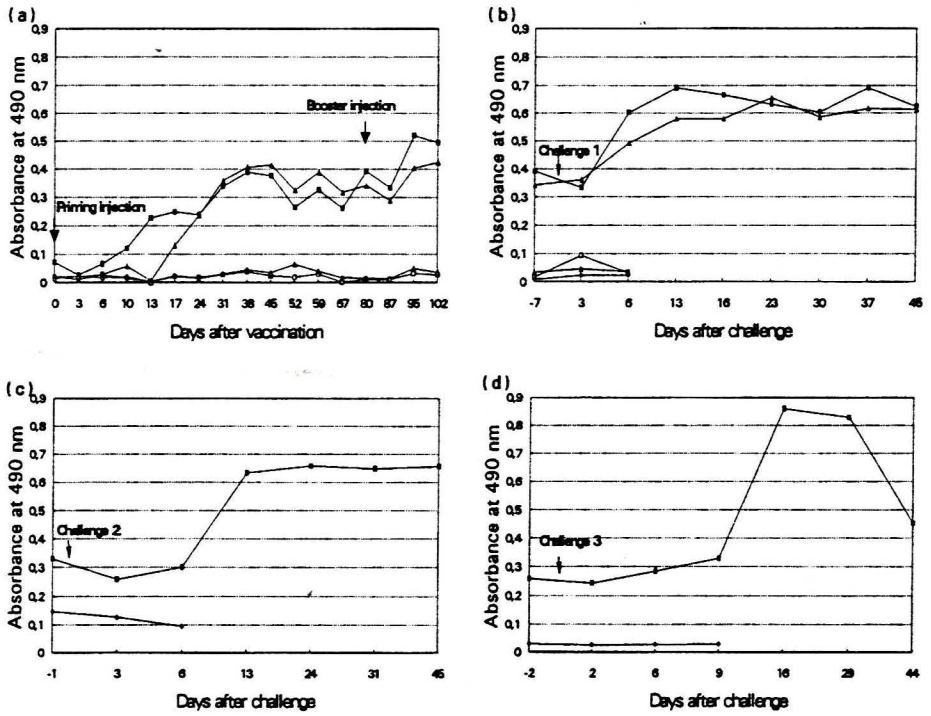


Fig. 1. Evolution of antibody titres measured by ELISA after immunisation of goats with (■) *Cowdria* antigen in ISA50, (□) PBS in ISA50, (▲) *Cowdria* antigen in Freund's adjuvant, (△) PBS in Freund's adjuvant, (◆) naive controls. (a) Vaccinal injections, (b) Challenge 1, 3.5 after boosting, (c) Challenge 2, 15 months after boosting, (d) Challenge 3, 17 months after boosting.

and a level of protection at least equal to that of FA (Castrucci et al., 1989; Roy et al., 1992; Ganne et al., 1994).

The duration of the protection induced by Montanide ISA50 was also investigated. Evidence of the establishment of a long lasting immunologic memory with this adjuvant was provided by the survival rates of 50% and 80% obtained 15 and 17 months following the booster injection (table 1). It was observed that the dose of *Cowdria* used for the challenge had an effect on the survival rate of the goats. Thus a dose giving an incubation period similar to that generally observed in natural infections led to a survival rate of 80%. The antibody response of the ISA 50 formulated vaccine persisted for up to 17 months, as described for many w/o formulated vaccines (Altman and Dixon, 1989). However, antibodies are of minor importance in protection against cowdriosis (Du Plessis, 1970; Martinez et al., 1994), and it is likely that the protection of goats was conferred via the induction of a cell mediated immune response. This type of immune response is of major

importance in the protection against intracellular parasites (Kaufman, 1995), an observation which has been confirmed in cowdriosis by adoptive immune transfer performed in a mouse model by Du Plessis et al. (1991).

The powerful immunostimulatory effect of w/o emulsions is mediated by the creation of a depot from where the antigen is slowly released, ensuring a continuous stimulation of the immune system, and the induction of a local inflammatory reaction consisting mainly of oil-ingesting macrophages and lymphocytes (Altman and Dixon, 1989). This local inflammatory reaction constitutes the major drawback in the use of oil adjuvants. In our study, animals reacted by a slight hyperthermia following immunisation and by the development of sterile abscesses at the site of injection. The reduction of the volume of adjuvant injected into animals (Anderson et al., 1971) and the use of the intra-muscular route for immunisation (Phan Thanh Phuong, 1992; Jackson and Opdebeeck, 1994) could help in solving this problem.

These improvements are necessary for a better acceptability of the vaccine by the farmers. However, the long-lasting protection and the good levels of protection obtained with a dose similar to that experienced by the animals in natural conditions suggest that a natural field challenge could efficiently boost the immune response of vaccinated animals without inducing heavy losses in small livestock. These results, associated with the fact that any stock of *Cowdria* can be incorporated in the vaccinal preparation to cover the antigenic repertoire of the microorganism, make this inactivated vaccine a first generation vaccine ready to be tested in the field.

Aknowledgements

This work was supported by a grant from the European Community under contract N° TS3-CT91-0007. We are very greatful to Dr Vincent Ganne from SEPPIC for providing Montanide ISA 50 adjuvant.

References

1. Altman, A., and Dixon, F.J., 1989. Immunomodifiers in vaccines. *Adv. Vet. Sci. Comp. Med.* 33, 301-343.

2. Anderson, E.C., Masters, R.C., Mowat, G.N., 1971. Immune response of pigs to inactivated foot-and-mouth disease vaccines. *Res. vet. Sci.* 12, 342-350.

3. Barré, N., Uilenberg, G., Morel, P.C. and Camus, E., 1987. Danger of introducing heartwater onto the American mainland: Potential role of indigenous and exotic *Amblyomma* ticks. *Onderstepoort J. Vet. Res.* 54, 405-417.

4. Bezuidenhout, J.D., Paterson, C.L. and Barnard, B.J.D., 1985. *In vitro* cultivation of *Cowdria ruminantium*. *Onderstepoort J. Vet. Res.* 52, 113-120.

5. Bezuidenhout, J.D., 1989. *Cowdria* vaccines, p. 31-42. In I.G. Wright, Veterinary protozoan and hemoparasite vaccines. Boca Raton, CRC Press, Inc.

6. Bradford, M.M., 1976. Microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Chem.* 72, 248-254.

7. Castrucci, G., Frigeri, F., Ferrari, M., Aldrovandi, V., Tassini, F., 1989. Further studies on passive immunization of newborn calves against rotaviral infection. *Comp. Immunol. Microbiol. infect. Dis.* 12, 71-76.

8. Cook, D.R., Hill, H.T., Kinker, D.R., 1990. Efficacy of a killed gpX deleted pseudorabies virus vaccine. *Can. J. Vet. Res.* 54, 438-445.

9. Du Plessis, J.L., 1970. Immunity to heartwater. I. A preliminary note on the role of serum antibodies. *Onderstepoort J. Vet. Res.* 37, 147-150.

10. Du Plessis, J.L., Berche, P. and Van Gas, L., 1991. T Cell-mediated immunity to *Cowdria ruminantium* in mice: the protective role of Lyt-2^+ T cells. *Onderstepoort J. Vet. Res.* 58, 171-179.

11. Edelman, R., 1980. Vaccine adjuvants. *Rev. Infect. Dis.* 2, 370-383.

12. Ganne, V., Eloit, M., Laval, A., Adam, M., Trouve, G., 1994. Enhancement of the efficacy of a replicative-defective adenovirus-vectored vaccine by the addition of oil adjuvants. *Vaccine* 12, 1190-1196.

13. Jackson, L.A., Opdebeeck, J.P., 1994. Quil A and ISCOMs as adjuvants for midgut membrane antigens of *Boophilus microplus*. *Appl. Parasitol.* 35, 87-98.

14. Jones, G.L., Spencer, L., Lord, R., Mollard, R., Pye, D., Saul, A., 1990. Peptide vaccines derived from a malarial antigen, effects of dose and adjuvants on immunogenicity. *Immunol. Lett.* 24, 253-260.

15. Jongejan, F., 1991. Protective immunity to heartwater (*Cowdria ruminantium* infection) is acquired after vaccination with *in vitro* attenuated Rickettsiae. *Infec. Immun.* 59, 729-731.

16. Jongejan, F., Vogel, S.W., Gueye, A., Uilenberg, G., 1993. Vaccination against heartwater using *in vitro* attenuated *Cowdria ruminantium* organisms. *Rev. Elev. Méd. Vét. Pays Trop.* 46, 223-227.

17. Jongejan, F., Uilenberg, G., 1994. Ticks and control methods. *Rev. sci. tech. Off. int. Epiz.* 13, 1201-1226.

18. Kaufman, H.E.S., 1995. Immunity to intracellular microbial pathogens. *Immunol. Today* 16, 338-342.

19. Mahan, S.M., Andrew, H.R., Tebele, N., 1995. Immunisation of sheep against heartwater with inactivated *Cowdria ruminantium*. *Res. Vet. Sci.* 58, 46-49.

20. Martinez, D., Swinkels, J., Camus, E. and Jongejan, F., 1990. Comparaison de trois antigènes pour le diagnostic de la cowdriose par immunofluorescence indirecte. *Rev. Elev. Méd. Vét. Pays Trop.* 43, 159-166.

21. Martinez, D., Coisne. S., Sheikboudou,.C. and Jongejan, F., 1993. Detection of antibodies to *Cowdria ruminantium* in the serum of domestic ruminants by ELISA. *Rev. Elev. Méd. Vét. Pays Trop.* 46, 115-120.

22. Martinez, D., Maillard, J.C., Coisne, S., Sheikboudou, C., Bensaid, A., 1994. Protection of goats against heartwater acquired by immunisation with inactivated elementary bodies of *Cowdria ruminantium*. *Vet. Immunol. Immunopathol.* 41, 153-163.

23. Perreau, P., P.C. Morel, N. Barré, and P. Durand. 1980. Existence de la cowdriose (heartwater) à *Cowdria ruminantium* chez les ruminants des Antilles françaises (La Guadeloupe) et des Mascareignes (La Réunion et île Maurice). *Rev. Elev. Méd. Vét. Pays Trop.* 33, 21-22.

24. Phan Thanh Phuong, 1992. Haemorragic septicaemia in Vietnam and its control approach. in *International Workshop on Pasteurellosis in Production Animal*, Bali, august 10-13.

25. Provost, A. and Bezuidenhout, J.D., 1987. The historical background and global importance of heartwater. *Onderstepoort J. Vet. res.* 54, 165-169.

26. Reed, L. and Muench, H., 1938. A simple method for estimating fifty percent endpoints. *Am. J. Hyg.* 27, 493-497.

27. Roy, P., French, T., Erasmus, B.J., 1992. Protective efficacy of virus-like particles for bluetongue disease. *Vaccine* 10, 28-32.

28. Totté, P., Blankaert, D., Zilimwabagabo, P. and Werenne, J., 1993. Inhibition of infectious yield of *Cowdria ruminantium* by interferons alpha and gamma in endothelial cells. *Rev. Elev. Méd. Vét. Pays Trop.* 46, 189-194.

29. Uilenberg, G., 1995. International collaborative research, significance of tick-borne hemoparasitic diseases to world animal health. *Vet. Parasitol.* 57, 19-41.

30. Uilenberg, G., Barré, N., Camus, E., Burridge, M.J. and Garris, G.I., 1984. Heartwater in the Caribbean. *Prev. Vet. Med.* 2, 255-267.

31. Uilenberg, G., Camus, E. and Barré, N., 1985. Quelques observations sur une souche de *Cowdria ruminantium* isolée en Guadeloupe (Antilles françaises). *Rev. Elev. Méd. Vét. Pays Trop.* 38, 34-42.

CHAPTER 7

Analysis of T-cell response in cattle immunized against heartwater by vaccination with killed elementary bodies of *Cowdria ruminantium*

P. Totté [a], D. McKeever [b], D. Martinez [c], A. Bensaid [a]

[a] *CIRAD-EMVT, BP 5035, 34032 Montpellier, France.*

[b] *ILRI, P.O. Box 30709, Nairobi, Kenya.*

[c] *Centre International en Recherche Agronomique pour le Développement, Département d'Elevage et de Médecine Vétérinaire des Pays Tropicaux (CIRAD-EMVT), BP 1232, 97184 Pointe à Pitre Cedex, Guadeloupe, West Indies.*

Infection and Immunity, 1997, 65 (1), 236-241.

Abstract

Cattle were successfully immunised against heartwater using a lysate of *Cowdria ruminantium* formulated in Freund's adjuvant. Vaccinated animals proved fully resistant to virulent challenge three and ten months after vaccination. For the first time a helper T lymphocyte response to *Cowdria* antigens was observed and characterised. *Cowdria*-specific T-cell lines generated from vaccinated animals by *in vitro* restimulation with *Cowdria* lysates are 95-100 % $CD4^+$, MHC class II-restricted and produce IFNγ. They proliferate in response to autologous monocytes infected with live *Cowdria* but not to uninfected monocytes. These T-cell lines will facilitate the search for *Cowdria* antigens that are immunogenic for T cells and therefore of relevance to the development of a subunit vaccine against the disease.

Introduction

Cowdria ruminantium is a tick-transmitted rickettsia that causes heartwater, or cowdriosis, an economically important disease of ruminants in sub-Saharan Africa and the Caribbean Islands (32).

The available method for vaccination against heartwater consists of administration of live virulent organisms to animals followed by tetracycline treatment during the febrile reaction (4). This infection and treatment method is cumbersome and risky and the search for safer, more practical, vaccines against the disease is therefore a priority (32). Attenuation of the pathogen has been reported (11), but the only available attenuated vaccine is based on the Senegalese strain of *C. ruminantium,* which does not cross-protect against several other strains (12). More recently, successful immunisation with inactivated *Cowdria* organisms emulsified in Freund's adjuvant has been achieved in goats (19) and sheep (16). This suggests that the development of a subunit vaccine against the disease may be feasible. However, identification of defined *Cowdria* antigens for use in a recombinant vaccine will depend on a better understanding of the mechanisms involved in protective immunity and in the pathogenesis of the disease.

Immunological studies on heartwater in ruminants have concentrated solely on antibody responses. In some instances hyperimmune sera from cattle and mice have been shown to neutralise the infection *in vitro* (6) whereas in others no significant effect was observed (19). Transfer of hyperimmune serum or purified gamma globulins did not confer protection in ruminants (7) or in mice (6). Since *C. ruminantium* is an obligate intracellular pathogen it is likely that T-cell-mediated immune mechanisms play a role in the protection. Studies in mice have shown that transfer of Lyt-2^+ T lymphocytes from animals immunised by infection and treatment protects susceptible recipients against *Cowdria* infection (8). There are no reports to date on T-cell responses to *Cowdria* infection in ruminants.

In this study, we confirm the feasibility of using killed elementary bodies of *Cowdria* to immunise cattle against heartwater. We have studied cellular aspects of the bovine immune response to *Cowdria* and report on the characterisation of a *Cowdria*- specific $CD4^+$ T lymphocyte response in immunised cattle. We also define a system whereby $CD4^+$ T-cell lines can be used to identify *Cowdria* antigens that are immunogenic for T lymphocytes.

Material and methods

Cowdria cultivation and antigen preparation

Cowdria ruminantium (Gardel stock) was grown in bovine umbilical endothelial cells (BUEC) as previously described (18). When the culture showed between 70 - 80 % cytopathic effect the supernatant was collected and cell debris was removed by

centrifugation at 1000 x g for 10 min. The supernatant was further centrifuged at 14000 x g for 30 min and the pellet was resuspended in 0.1 M PBS, pH 7.1. *Cowdria* elementary bodies were inactivated by 5 freeze/thaw cycles using liquid nitrogen and stored at -20 °C until used for immunisation. The resulting preparation was not infective for BUEC, indicating that it did not contain viable organisms (data not shown). For T-cell proliferation assays, *Cowdria* antigens were prepared as above but after centrifugation at 14000 x g for 30 min the pellet was passed ten times through a 26 gauge needle and resuspended in sucrose-phosphate-glutamate buffer (5). Organisms were further purified on discontinuous Renografin density gradients (33). After three washes in PBS, purified organisms were freeze/thawed 5 times in liquid nitrogen and stored as described above. Lysates of uninfected BUEC were also prepared for control purposes. The protein content of lysates was determined by the Bradford method (Pierce).

Immunisation and challenge of animals

Nine 8 month old Ayrshire cattle were obtained from the ILRI ranch, which practises regular spraying of animals with acaricides. Pre-bleed sera of all animals were negative for anti-*Cowdria* antibodies as determined by an IFA test (18). Five animals were immunised by intramuscular inoculation of 0.5 ml of killed elementary bodies of *C. ruminantium* formulated in an equal volume of complete Freund's adjuvant (CFA). One month later, the calves received a similar inoculation in incomplete Freund's adjuvant (IFA). Each animal received a total of approximately 100 μg of killed *Cowdria*. Two animals were challenged three months after boosting (group A) and three after 10 months (group B), by i.v. injection of 3 ml of a virulent *Cowdria* preparation derived from *in vitro* cultures. This dose had been shown previously to cause 100 % mortality in naive cattle and corresponded to 135 TCLD50 as determined by the 50 % culture lytic dose titration method (28). The remaining four animals were used as non-immunised controls. Previous experiments have shown that neither Freund's adjuvant nor inactivated *Cowdria* alone confer protection in small ruminants against virulent challenge with the agent (20, 16).

Generation of short-term T-cell lines

Peripheral blood mononuclear cells (PBMC) were collected from group B animals eight months after the primary immunisation, two months before challenge. Cells were separated by flotation of blood collected in Alsever's solution on Ficoll/Paque (Pharmacia, Uppsala, Sweden). The cells were cultured in 24 well plates, at a density of 1 x 10^6/ml, in RPMI medium containing 10 % fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), 5 x 10^{-5} M 2-

mercaptoethanol (complete medium) and 1 μg/ml of *Cowdria* lysate, in a total volume of 2 ml. After culture for 7 days at 37 °C in a humidified atmosphere of 5 % CO_2 in air, viable cells were restimulated with *Cowdria* lysate at a density of 5 x 10^5 cells/well in complete medium, in the presence of 1 x 10^6 autologous γ-irradiated (500 rads) PBMC as antigen presenting cells (APC). Viable cells were cryopreserved in liquid nitrogen one week after the last restimulation. Freshly thawed cells were used in T-cell proliferation assays only if their viability was above 90 %.

T-cell proliferation assays

Fresh PBMC were seeded at a density of 3 x 10^5 cells/well in 96-well flat bottomed plates, in a final volume of 200 μl. For assays with cell lines, wells were seeded with 2 x 10^4 T cells and 6 x 10^4 irradiated syngeneic PBMC. Where appropriate, *Cowdria* antigens were included in cultures at a previously determined optimal concentration of 1 μg/ml. On day four of the culture period, 0.5 μCi of [^{125}I]iododeoxyuridine (Amersham International, Amersham, U.K.) was added to each well and incubated overnight. The cells were harvested onto glass fiber filters using a cell harvester (Flow laboratories) and radioactivity counted using a Beckman 5500 gamma counter. Results are expressed as counts per minute (CPM) averaged from duplicate wells. In some experiments monocytes infected with live *Cowdria* were used as APC. Monocytes were purified from PBMC by adherence to polystyrene (10) and 3 x 10^6 cells were infected with the equivalent of 650 $TCLD_{50}$ (28) of freshly prepared elementary bodies of *Cowdria*. The cells were harvested 48 h. after infection, fixed in 0.1 % glutaraldehyde and used as APC (1 x 10^4 cells/well) in proliferation assays. Electron micrographic studies confirmed that, at the time of harvest, *Cowdria* organisms had differentiated into reticulate bodies within infected monocytes (manuscript in preparation). For blocking experiments, monoclonal antibodies (Mab) specific for bovine class I and class II MHC molecules were added to the assay in the form of ascites at a final dilution of 1/500. Mab IL-A88 (IgG2a) recognises a monomorphic determinant on bovine class I MHC molecules (31), mab IL-A21 (IgG2a) is specific for class II MHC DR (9) and mab VC9 recognises both class II MHC DQ and DR subtypes (30, 3).

FACS analysis

The surface phenotype of PBMC and T-cell lines was analysed by indirect immunofluorescence staining using specific monoclonal antibodies (Mab). Mabs IL-A12 (1), IL-A51 (14) and GB21A (15) recognise bovine CD4, CD8 and γδ T cell receptor respectively. Mab IL-A30 (34) recognises bovine cell surface IgM and

TABLE 1. Outcome of the homologous virulent challenge of vaccinated and naive cattle

Animal	Treatment date (mo/yr)		Days to fever[a]	Maximum temp (°C)	Time to death (days)	Outcome
	Primary immunization	Challenge				
Immunized						
Group A						
322	5/94	8/94	14	40.2	—[b]	Immune
338	5/94	8/94	8	40.7	—	Immune
Group B						
295	5/94	3/95	5	41.0	—	Immune
296	5/94	3/95	5	40.9	—	Immune
304	5/94	3/95	3	41.3	—	Immune
Naive						
109		7/94	13	40.7	16	Death
110		7/94	14	40.3	18	Euthanized[c]
294		8/94	7	41.0	14	Euthanized
264		3/95	8	40.5	12	Death

[a] Number of days between infection and pyrexia (T > 39.5°C).
[b] —, death did not occur.
[c] Euthanized upon appearance of nervous signs.

therefore defines B cells. Mab IL-A111 recognises the bovine IL-2 receptor (22). Mab J11 (2) recognises a monomorphic determinant on bovine class II MHC molecules. Cells were stained as described by MacHugh et al (14) and analysed on a fluorescence-activated cell sorter (FACS II, Becton Dickinson, Sunnyvale, CA, USA). Results are expressed as % of total cells that fluoresced above background level.

Bovine IFNγ assay

Bovine IFNγ in culture supernatants was detected using a specific ELISA kit (CSL, Parkville, Australia) and quantified against a recombinant bovine IFNγ (CIBA-GEIGY). Assays were performed on day 4 supernatants, which were observed in pilot experiments to reflect maximal expression.

Results

Clinical response of cattle to Cowdria challenge

All immunised animals survived virulent challenge (Table 1), showing no clinical signs other than transient fever. In contrast, all naive challenge control animals developed heartwater and either died without overt clinical signs (peracute heartwater) or were euthanised upon appearance of nervous signs ranging from inco-ordination to lateral recumbency with opisthotonus (acute heartwater). Brain smears prepared from all naive animals at autopsy were positive for colonies of *Cowdria*. No significant difference was observed between immunised and naive animals in the duration nor in the magnitude of the febrile reaction.

Characterisation of the proliferative response to Cowdria antigens (Ag)

When tested prior to challenge, PBMC from all immunised animals responded strongly to *Cowdria* Ag. Proliferative responses of PBMC taken during the course of immunisation from animals 338 and 295, which are representative individuals from group A and B are illustrated in fig.1. No proliferation was observed prior to immunisation or in naive controls. PBMC from *Cowdria*-immunised animals responded to uninfected BUEC Ag but the proliferation was consistantly lower than that response observed with *Cowdria* Ag. This was attributed to the fact that the animals were immunised with partially purified *Cowdria* Ag, which undoubtedly also contained BUEC antigens. Boosting inoculation did not significantly increase the level of the response to *Cowdria* Ag.

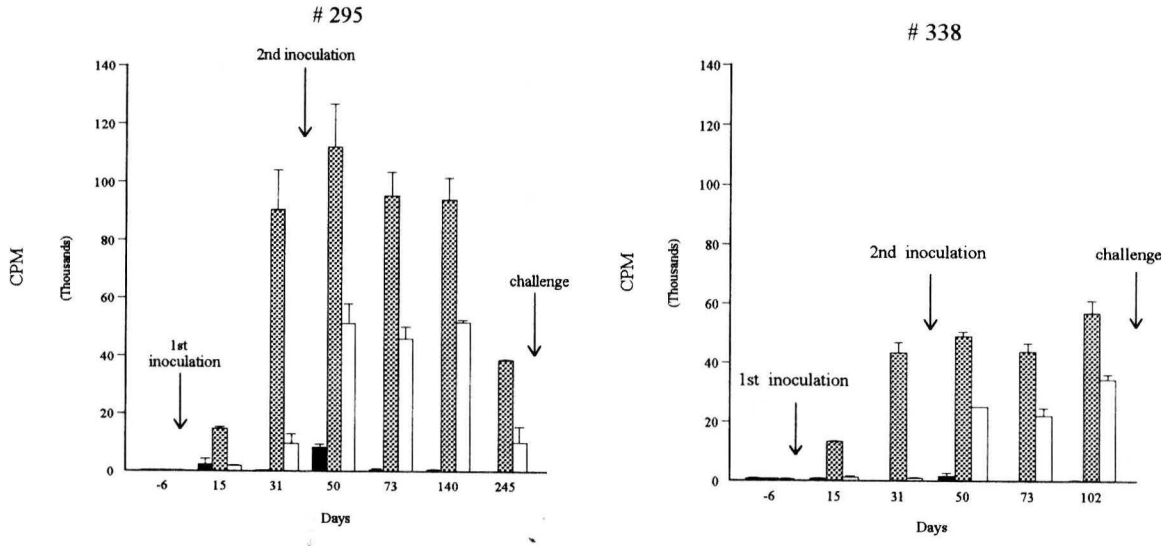


Fig. 1. Proliferative response of PBMC from two *Cowdria*-immunized cattle (295 and 338). PBMC were incubated with medium (black bars), inactivated *Cowdria* (grey bars), and uninfected BUEC lysate (white bars). Both *Cowdria* and BUEC Ag were used at a final concentration of 1 μg ml^{-1}. The results represent means plus standard deviations of duplicate cultures.

The phenotype of responding lymphocyte populations was determined by FACS analysis after five days incubation with the different Ag preparations. Incubation of PBMC with *Cowdria* Ag resulted in an increase in relative cell numbers in only the $CD4^+$ fraction (Table 2). The proportion of blasts also dramatically increased in the $CD4^+$ fraction as well as in other populations but to a lesser extent and for the latter without any increase in relative numbers. In contrast, when PBMC were incubated with BUEC Ag, the majority of responding cells were γδ T lymphocytes (data not shown). The expression of class II MHC molecules and IL-2 receptors on the surface of T cells responding to the different stimuli was also examined (Table 2). An increase in the expression of both class II MHC and the IL-2 receptor was observed only in the $CD4^+$ fraction after incubation with *Cowdria* Ag, which is consistent with an active proliferative response. Other lymphocyte populations in the culture also expressed higher levels of class II MHC molecules but did not up-regulate IL-2 receptor expression. This is in line with the observation that a proportion of CD8+ and γδ T cells were enlarged but did not proliferate in response to *Cowdria* Ag. Increased expression of class II MHC and the IL-2 receptor after stimulation of PBMC with BUEC Ag was observed in γδ T cells only (data not shown). Although FACS analysis revealed an unexpectedly high proportion of sorted $CD4^+$ T cells expressing class II MHC molecules after culture in medium alone (Table 2), the level of fluorescence was low in comparison to cells incubated with *Cowdria* Ag.

Generation and characterisation of Cowdria-specific T-cell lines

After three restimulations at weekly intervals with *Cowdria* lysate, cell lines proliferated in response to *Cowdria* Ag but not to BUEC Ag (Table 3). They also recognised autologous monocytes infected with the live pathogen (Table 3). When analysed one week after the third restimulation, these *Cowdria* - specific short term cell lines were 95 - 100 % $CD4^+$ T lymphocytes as shown by FACS analysis, with the remaining cells being $CD8^+$ (Fig. 2). Maximal proliferation occurred at day 4 and required the presence of autologous irradiated PBMC as APC. No proliferation was observed when heterologous PBMC were used as APC, suggesting that the response was not the result of a mitogenic factor (data not shown). Blocking experiments using monoclonal antibodies confirmed that proliferation was MHC class II-restricted (Table 4). A combination of two anti-MHC class II antibodies was necessary to completely abrogate the proliferation suggesting that both DQ and DR subtypes of MHC class II are involved.

Upon stimulation with *Cowdria* Ag, these T-cell lines secreted significant levels of IFNγ into the medium (Table 5). Although we cannot exclude the possibility that contaminating $CD8^+$ T cells also produced IFNγ in these cultures,

TABLE 2. T-cell phenotype and Class II MHC and IL-2 receptor surface expression after 5 days of culture with medium and *Cowdria* antigens

Animal no.	Days after boosting	Cell subpopulations (% total PBMC)[a]						CII[b,c] and IL-2R[d] expression (% positive cells)											
		CD8		CD4		γδ		CD8				CD4				γδ			
								M		CAg		M		CAg		M		CAg	
		M[e]	CAg[f]	M	CAg	M	CAg	CII	IL-2R	CII	IL-2R	CII	IL-2R	CII	IL-2R	CII	IL-2R	CII	IL-2R
295	140	12	10	28	42	22	20	13	8	23	4	20	13	90	31	8	8	28	10
		26	**45**	**20**	**78**	**23**	—[g]												
296	140	18	18	30	44	24	28	20	6	35	5	17	6	64	25	12	6	39	6
		20	**57**	**10**	**73**	**12**	**34**												
304	140	20	14	25	38	26	23	26	3	23	1	23	10	80	38	29	7	38	8
		40	**46**	**20**	**76**	**22**	**56**												
322	33	22	15	22	38	16	17	—	—	—	—	30	15	83	50	—	—	—	—
		30	**71**	**33**	**84**	**50**	**76**												
338	33	16	17	20	39	16	20	—	—	—	—	15	8	52	29	—	—	—	—
		25	**66**	**22**	**82**	**22**	**52**												

[a] Boldface numbers, % lymphoblasts of a given phenotype.
[b] CII, class II MHC.
[c] Class II MHC-positive cells in control cultures were expressed at low levels.
[d] IL-2R, IL-2 receptor.
[e] M, medium.
[f] CAg, *Cowdria* Ag.
[g] —, not done.

TABLE 3. Specificity of *Cowdria*-responsive T-cell lines

Cell line and no. of restimulations	Proliferative response (cpm)[a]				
	Medium	*Cowdria* lysate	BUEC	Monocytes −[b]	Monocytes +[c]
295					
0	555 ± 66	38,764 ± 177	10,080 ± 5,448	—[d]	—
3	1,080 ± 102	53,584 ± 1,869	601 ± 30	1,198 ± 282	28,438 ± 2,394
296					
0	657 ± 26	26,665 ± 4,924	6,401 ± 1,158	—	—
3	1,290 ± 880	19,836 ± 1,628	949 ± 343	1,210 ± 97	17,715 ± 713
304					
0	213 ± 118	20,623 ± 3,279	6,925 ± 1,485	—	—
3	965 ± 262	40,689 ± 3,742	896 ± 100	—	—

[a] Results are means ± standard deviations of duplicate cultures.
[b] Monocytes −, uninfected monocytes.
[c] Monocytes +, monocytes infected with live *Cowdria*.
[d] —, not done.

cell line 296, which had the highest percentage of $CD8^+$ T cells, was the lowest IFNγ producer.

Discussion

We have confirmed the feasibility of immunising cattle against heartwater using killed *Cowdria* organisms. With a total of five cattle being solidly protected against homologous challenge, three survived a lethal challenge ten months after immunisation. Immunised animals showed no signs of illness after challenge other than an early febrile reaction. This method of vaccination has also protected goats (19) and sheep (16) and is a significant improvement on the infection and treatment technique used to date. No tetracycline treatment is required and all inoculations are intramuscular, abrogating the requirement for trained veterinarians to perform the immunisations. In addition, because it is based on killed *Cowdria* organisms it can be used in areas where the disease has spread but is not yet endemic. Finally, because the vaccine is produced *in vitro* in endothelial cell cultures, the risk of it transmitting other diseases is considerably reduced. Efforts are currently focused on the transfer of this method from the laboratory to the field. This will undoubtedly be hastened by the observation that CFA, which is unacceptable for use in food animals, can be substituted with an acceptable, commercially available adjuvant without loss of protection in goats (20). Success of this immunisation method in the field will, however, depend on its capacity to protect against heterologous challenge, or on whether a cocktail of antigenically different strains of *Cowdria* can provide comprehensive protection. It will also be necessary to determine the optimal dose of killed *Cowdria* organisms required for protection against tick-delivered challenge. Although it is likely that the dose of inactivated *Cowdria* can be reduced, large quantities of endothelial cells will still be needed to produce it. This might be achieved through the culture of endothelial cells on collagen microspheres in bioreactors (26). In the longer term, however, the use of recombinant *Cowdria* proteins produced by transformed bacteria is a more practical solution. Reagents generated in the course of this study provide a solid basis for the identification of immunoprotective Ag of *Cowdria*. Available information on immunity to heartwater and intracellular pathogens in general would suggest that cellular immune responses play an important role in protection. Previous immunological studies on heartwater in ruminants have focused entirely on antibody responses; we have now investigated the capacity of cell-mediated responses to protect against the agent.

We observed that PBMC from vaccinated animals proliferated vigorously in response to *Cowdria* Ag *in vitro*. No proliferation was observed before vaccination or in naive controls. *Cowdria* Ag preferentially stimulated immune $CD4^+$ T lymphocytes to proliferate. Stimulation of $CD4^+$ T cells was dependent on irradiated APC and exogenous growth factors were not required. Responding cells expressed

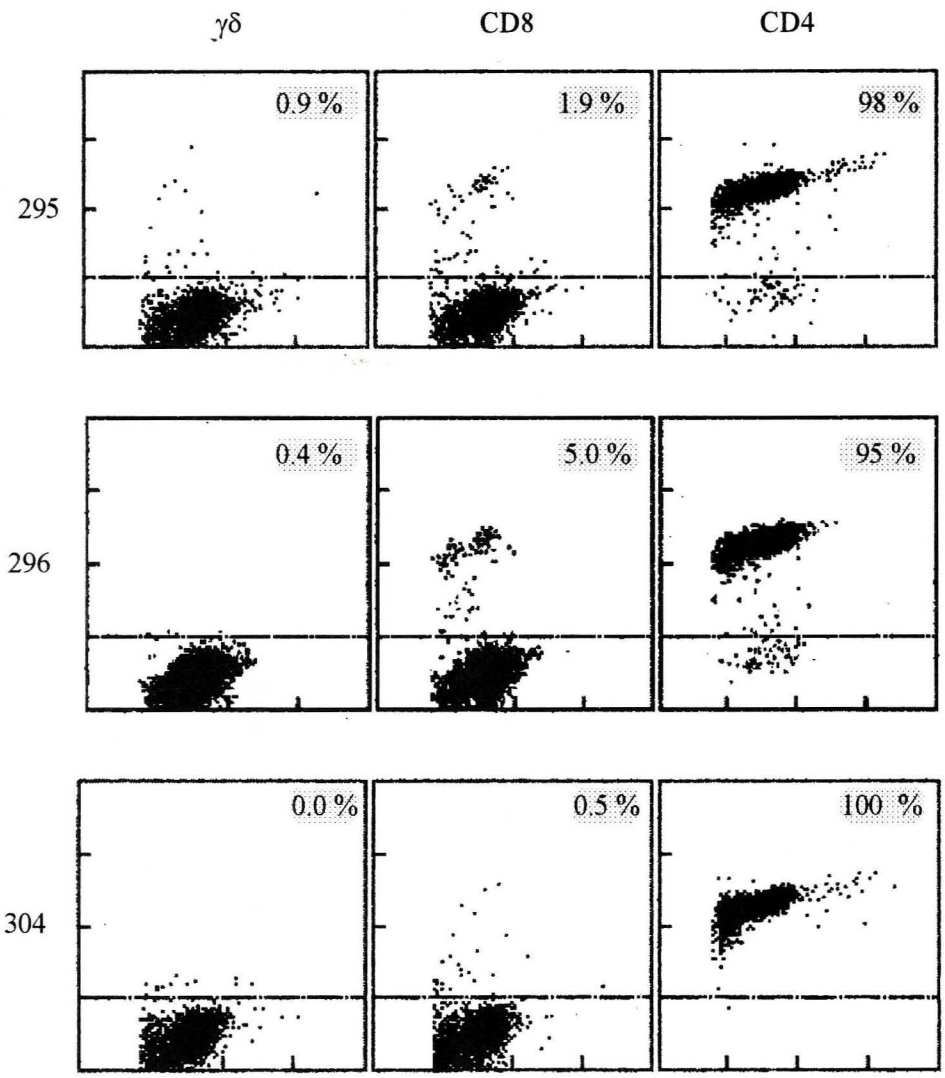


Fig. 2. Phenotypes of bovine *Cowdria*-specific T-cell lines. FACS analysis was performed with specific MAb against bovine CD4, CD8, and γδ T-cell receptor. The y axis represents the intensity of fluorescence (log scale) and the x axis represents cell size (forward scatter, linear scale). Results are expressed as the percentage of total cells above background level (indicated by the horizontal line) that fluoresced.

high levels of class II MHC molecules and the IL-2 receptor on their surface. Repeated stimulation of PBMC with *Cowdria* lysates allowed enrichment of this cell type in short term T-cell lines. Unlike freshly prepared immune PBMC, these lines did not respond to uninfected BUEC Ag and were therefore entirely specific for *Cowdria* Ag. They also proliferated in response to autologous monocytes infected with live Cowdria organisms. This observation may have significance for the activation of these responses during challenge. Although there is no evidence that *Cowdria* replicates within monocytes/macrophages *in vivo*, extracellular *Cowdria* organisms are found in the blood of infected animals (23) and, therefore, are available for phagocytosis by monocytes and presentation to the immune system.

Table 4
Effect of MHC-specific mAb on proliferation of bovine *Cowdria*-specific T-cell lines.

Cell line	Specificity of blocking mAb	T-cell response (cpm)[a]	
		Cowdria Ag -[b]	*Cowdria* Ag +[c]
295	medium	4100 ± 512	64224 ± 625
	class I	-	67445 ± 1025
	class II DQ+DR (1)	-	33519 ± 2153
	class II DR pan (2)	-	28906 ± 962
	(1) + (2)[e]	-	8333 ± 176
296	medium	2860 ± 900	23598 ± 1333
	class I	-	24192 ± 2887
	class II DQ+DR (1)	-	15040 ± 705
	class II DR pan (2)	-	10577 ± 911
	(1) + (2)	-	4032 ± 446

[a] results are mean cpm ± standard deviations of duplicate experiments
[b] *Cowdria* Ag -
[c] *Cowdria* Ag +
[d] -, not done.
[e] (1) + (2), class II DQ + DR and class II DR pan

Induction of a $CD4^+$ T-cell response to *Cowdria* Ag by immunisation with killed *Cowdria* formulated in CFA was predictable since inactivated or lysed organisms are likely to be processed for presentation in association with MHC class II molecules. Nonetheless, class I MHC-restricted $CD8^+$ T cell responses to immunisation with exogenous, nonreplicating agents and soluble antigens have been

reported (25, 25). The possibility that this is the case in animals immunised with inactivated *Cowdria* is currently beeing investigated.

Although the mechanisms that conferred protection in these animals have not yet been determined, our results are consistent with the involvement of $CD4^+$ T lymphocytes. This population could affect *Cowdria* infection at different levels. Maturation of antibody responses is known to require the input of helper T lymphocytes. It has been shown previously that animals immunised with inactivated *Cowdria* in CFA have higher antibody titers than those vaccinated with inactivated organisms only (16), although a separate study revealed that antibody levels were similar between protected and non protected vaccinated animals (19). Whether the quality rather than the quantity of antibodies produced is important for protection has yet to be investigated. $CD4^+$ T cells may also provide help for the generation of cytotoxic $CD8^+$ T lymphocytes by secreting stimulatory factors and/or through cell contact (13, 21). This observation has recently been confirmed in cattle infected with the intracellular protozoan *Theileria parva* (24). Finally, $CD4^+$ T lymphocytes could also contribute to protection through the release of cytokines such as IFNγ. We have reported previously that recombinant bovine IFNγ is a potent inhibitor of *Cowdria* growth both *in vitro* (27,30) and *in vivo* (29). Endogenous IFNγ produced by con A stimulated PBMC was also found to inhibit the growth of *Cowdria in vitro* (17).

Table 5
IFNγ production by *Cowdria*-specific T-cell lines[a].

Cell line	IFNγ production (ng/ml)[b]	
	-	+
295	< 0.02	12.5 ± 2
296	< 0.02	2.5 ± 1
304	< 0.02	13.8 ± 3

[a] T-cell lines were cultured for 4 days in the presence (+) or absence (-) of inactivated *Cowdria*.
[b] Values are means ± (where appropriate) standard deviations.

We have now established that *Cowdria* Ag can specifically induce $CD4^+$ T lymphocytes to produce IFNγ. The availability of *Cowdria*-specific $CD4^+$ T cell lines will now enable us to identify components of the agent that provoke these responses. These antigens would have clear potential for the development of a subunit vaccine against the disease.

In summary, we have shown that PBMC from animals rendered resistant to *Cowdria* challenge by vaccination with inactivated organisms contain *Cowdria*-specific, MHC class II restricted, IFNγ producing, $CD4^+$ T lymphocytes. These cells proliferate in response to autologous monocytes infected *in vitro* with live *Cowdria*. It remains to be shown whether this population also responds *in vivo* to live *Cowdria* challenge. This is currently being addressed through the use of lymphatic cannulation techniques in immune animals under challenge.

Acknowledgements

This work was partially funded by the French Ministry for Foreign Co-operation. We thank John Kamau for expert technical assistance. This is ILRI publication no. 96034.

References

1. Baldwin, C.L., A.J. Teale, J.G. Naessens, B.M. Goddeeris, N.D. MacHugh, and W.I. Morrison. 1986. Characterization of a subset of bovine T lymphocytes that express BoT4 by monoclonals and function, similarity to lymphocytes defined by human T4 and murine L3T4. *J. Immunol.* 136, 4385-4391.

2. Baldwin, C.L., W.I. Morrison, and J. Naessens. 1987. Differentiation antigens and functional characteristics expressed by bovine leukocytes. In Miyasaka M., and Z. Trnka (eds), comparative aspects of differentiation antigens in lympho-haemopoietic tissues, 445 - 498.

3. Ballingal, K.T. (ILRI). Personal communication.

4. Bezuidenhout, J.D. 1989. *Cowdria* vaccines. *In* Wright I.G. (ed.), Veterinary Protozoan and Hemoparasite vaccines. CRC Press, Inc., Boca Raton, Florida.

5. Bovarnick, M.R., J.C. Miller, and J.C. Snyder. 1950. The influence of certain salts, amino acids, sugars, and proteins on the stability of rickettsiae. *J. Bacteriol.* 59, 509-522.

6. Byrom, B., S.M. Mahan, and A.F. Barbet. 1993. The development of antibody to *Cowdria ruminantium* in mice and its role in heartwater disease. *Rev. Elev. Méd. Vét. Pays Trop.* 46, 197-201.

7. Du Plessis, J.L. 1970. Immunity to heartwater. I. A preliminary note on the role of serum antibodies. *Onderstepoort J. Vet. Res.* 37,147-150.

8. Du Plessis, J.L., P. Berche, and L. Van Gas. 1991. T cell-mediated immunity to *Cowdria ruminantium* in mice, the protective role of LYT-2+ cells. *Onderstepoort J. Vet. Res.* 58, 171-179.

9. Fraser, D.C., S. Craigmile, J.D.M. Campbell, R.A. Oliver, D.J. Brown, G.C. Russell, R.L. Spooner, and E.J. Glass. 1996. Functional expression of a cattle MHC class II DR-like antigen on mouse L cells. *Immunogenetics* 43, 296-303.

10. Goddeeris, B.M., C.L. Baldwin, O. ole-MoiYoi, and W.I. Morrison. 1986. Improved methods for purification and depletion of monocytes from bovine peripheral blood mononuclear cells. *J. Immunol. Meth.* 89, 165-173.

11. Jongejan, F. 1991. Protective immunity to heartwater (*Cowdria ruminantium* infection) is acquired after vaccination with *in vitro*-attenuated rickettsiae. *Infect. Immun.* 59, 3243-3246.

12. Jongejan, F., S.W. Vogel, A. Gueye, and G. Uilenberg. 1993. Vaccination against heartwater using *in vitro* attenuated *Cowdria ruminantium* organisms. *Rev. Elev. Méd. Vét. Pays Trop.* 46, 223-227.

13. Keene, J., and J. Forman. 1982. Helper activity is required for the *in vivo* generation of cytotoxic lymphocytes. *J. Exp. Med.* 155, 768-782.

14. MacHugh, N.D., A. Bensaid, C.J. Howard, W.C. Davis, and W.I. Morrison. 1991. Analysis of the reactivity of anti-bovine CD8 monoclonal antibodies with cloned T cell lines and mouse L-cells transfected with bovine CD8. *Vet. Immunol. Immunopathol.* 27, 169-172.

15. MacHugh, N.D., M.J. Hamilton, C.R. Wyatt, J.A. Orden, and W.C. Davis. A monoclonal antibody recognising the bovine TCR δ chain defines two populations of γδ T cells. (Submitted for publication)

16. Mahan, S.M., H.R. Andrew, N. Tebele, M.J. Burridge, and A.F. Barbet. 1995. Immunisation of sheep against heartwater with inactivated *Cowdria ruminantium*. *Res. Vet. Sci.* 58, 46-49.

17. Mahan, S.M., M. Sileghem, G.E. Smith, and B. Byrom. Neutralisation of bovine concanavalin A T cell supernatant-mediated anti-*Cowdria* activity with antibodies specific to interferon gamma but not to tumor necrosis factor. *Parasite Immunol.* 18, 317-324.

18. Martinez, D., J. Swinkels, E. Camus, and F. Jongejan. 1990. Comparaison de trois antigènes pour le diagnostic de la cowdriose par immunofluorescence indirecte. *Rev. Elev. Méd. Vét. Pays Trop.* 43, 159-166.

19. Martinez, D., J.C. Maillard, S. Coisne, C. Sheikboudou, and A. Bensaid. 1994. Protection of goats against heartwater is acquired by immunisation with inactivated elementary bodies of *Cowdria ruminantium*. *Vet. Immunol. Immunopathol.* 41, 153-163.

20. Martinez, D., J.M. Perez, C. Sheikboudou, A. Debus, and A. Bensaid. Comparative efficacy of Freund's and Montanide ISA50 adjuvants for the immunisation of goats against heartwater with inactivated *Cowdria ruminantium*. *Vet. Parasitol.* (in press)

21. Mizuochi, T., H. Golding, A.S. Rosenberg, L.H. Glimcher, T.R. Malek, and A. Singer. J. 1985. Both $L3T4^+$ and $Lyt2^+$ helper T cells initiate cytotoxic lymphocyte responses against major histocompatibility antigens but not against trinitrophenyl modified self. *J. Exp. Med.* 162, 427-443.

22. Naessens, J., M. Sileghem, N. MacHugh, Y.H. Park, W.C. Davis, and P. Toye. 1992. Selection of BoCD25 monoclonal antibodies by screening mouse L cells transfected with the bovine p55-interleukin-2 (IL-2) receptor gene. *Immunology* 76, 305-309.

23. Pienaar, J.G. 1970. Electron microscopy of *Cowdria ruminantium* in the endothelial cells of the vertebrate host. *Onderstepoort J. Vet. Res.* 37, 67-78.

24. Taracha, E.L.N., and D.J. McKeever. 1993. Inductive requirements of *Theileria parva*-specific cytotoxic T lymphocytes, p. 109-111. *In* D.J. McKeever (ed.), Novel immunization strategies against protozoan parasites. ILRI, Kenya.

25. Tevethia, S., D.C. Flyer, and R. Tijan. 1980. Biology of simian virus 40 (SV40) transplantation antigen (TrAg). *Virology* 107, 13-23.

26. Totté, Ph., D. Blankaert, T. Marique, C. Kirkpatrick, J.P. Van Vooren. 1993a. Bovine and human endothelial cells growth on collagen microspheres and their infection with the rickettsia *Cowdria ruminantium*, prospects for cells and vaccine production. *Rev. Elev. Méd. Vét. Pays Trop.* 46,153-155.

27. Totté, Ph., D. Blankaert, P. Zilimwabagabo, and J. Wérenne. 1993b. Inhibition of *Cowdria ruminantium* infectious yield by interferons alpha and gamma in endothelial cells. *Rev. Elev. Méd. Vét. Pays Trop.* 46, 189-194.

28. Totté, Ph., F. Jongejan, A.L. de Gee, and J. Wérenne. 1994a. Production of alpha interferon in *Cowdria ruminantium*-infected cattle and its effect on infected endothelial cell cultures. Infect. Immun. 62, 2600-2604.

29. Totté, Ph., N. Vachiéry, H. Heremans, A. Billiau, and J. Wérenne. 1994b. Protection against *Cowdria ruminantium* infection in mice with gamma interferon produced in animal cells, p. 595-599. *In* T. Kobayashi, Y. Kitagawa, and K. Okumura (ed.), Animal cell technology, basic and applied aspects, vol. 6. Proceedings of the Sixth International Meeting of the japanese Association for Animal Cell Technology, Kluwer Academic Publishers, Dordrecht, The Netherlands.

30. Totté, Ph., N. Vachiéry, D. Martinez, I. Trap, K.T. Ballingall, N.D. MacHugh, A. Bensaid, and J. Wérenne. 1996. Recombinant bovine interferon gamma inhibits the growth of *Cowdria ruminantium* but fails to induce MHC class II following infection of endothelial cells. *Vet. Immunol. Immunopathol.* 53, 61-71.

31. Toye, P.G., N.D. MacHugh, A.M. Bensaid, S. Alberti, A.J. Teale, and W.I. Morrison. 1990. Transfection into mouse L cells of genes encoding two serologically and functionally distinct bovine class I MHC molecules from a MHC-homozygous animal, evidence for a second class I locus in cattle. *Immunology* 70, 20-26.

32. Uilenberg, G. 1983. Heartwater (*Cowdria ruminantium* infection), current status. *Adv. Vet. Sci. Comp. Med.* 27, 427-480.

33. Weiss, E., J.C. Coolbaugh, and J.C. Williams. 1975. Separation of viable *Rickettsia typhi* from yolk sac and L cell host components by Renografin density gradient centrifugation. *Appl. Microbiol.* 30, 456-463.

34. Williams, D.J.L., J. Newson, and J. Naessens. 1990. Quantitation of bovine immunoglobulin isotypes and allotypes using monoclonal antibodies. *Vet. Immunol. Immunopathol.* 24, 267-283.

35. Yamada, A., M.R. Ziese, and J.F. Young. 1985. Influenza virus hemagglutinin-specific cytotoxic T cell response induced by polypeptide produced in *Escherichia coli*. *J. Exp. Med.* 162, 663-674.

CHAPTER 8

Flow cytometry analysis of peripheral blood mononuclear cell populations in cattle and goats immunised with inactivated *Cowdria ruminantium* in Freund's or Montanide ISA 50 adjuvants

D. Martinez [a], P. Totté [a], J.M. Perez [a], I. Trap [b], J. Kamau [c], C. Sheikboudou [a], N. Vachiery [b], D. MacKeever [c], A. Bensaid [b]

[a] *Centre International en Recherche Agronomique pour le Développement, Département d'Elevage et de Médecine Vétérinaire des Pays Tropicaux (CIRAD-EMVT), BP 1232, 97184 Pointe à Pitre Cedex, Guadeloupe, West Indies.*

[b] *CIRAD-EMVT, BP 5035, 34032 Montpellier, France.*

[c] *ILRI, P.O. Box 30709, Nairobi, Kenya.*

Draft

Abstract

Goats and cattle were immunised against heartwater with inactivated *Cowdria ruminantium* in an oil adjuvant, and challenged with a lethal dose of *C. ruminantium* at different times after vaccination. Flow cytometry was used to follow changes in peripheral blood mononuclear cell populations (PBMC) during vaccination and infection. No significant changes were noticed after vaccination and boosting. In contrast, after challenge, three main changes were observed reproducibly in goats and cattle, irrespective of the adjuvant used. A progressive depletion of $CD4^+$, $CD8^+$, and γδ T cell subsets during the first 10 days led to a lymphopenia. Goats which survived had a smaller decrease of their lymphocyte populations than animals that died. During the first two weeks, monocyte numbers increased greatly and monocytes had an activated aspect in all animals. This suggests an important role of these cells in the early immune to *C. Ruminantium* infection. The most stricking change was a considerable rise of the number of $CD8^+$ T lymphocytes which reached levels above 50% of the circulating PBMC at day 16 to 19 post infection. The functional analysis of these cells is underway and similar studies on leucocytes in other compartment of the host, e.g. in lymph nodes and the spleen, are also being considered.

Introduction

Cowdria ruminantium, the causative agent of heartwater, is a tick transmitted rickettsia affecting both wild and domestic ruminants. It is present in sub-Saharan Africa, Madagascar and several Caribbean islands (Provost and Bezuidenhout, 1987; Uilenberg, 1983). It is an obligate intracellular bacterium which preferentially infects endothelial cells and can be found in neutrophils during the febrile phase of the disease. Economic losses are important in endemic regions (Uilenberg, 1995) where there is a need for efficient and safe vaccines. The method of immunisation currently used in the field is still the infection and treatment procedure described soon after the discovery of efficient chemotherapeutic agents (Neitz and Alexander, 1945). The acquired immunity is strong and long lasting but the method is risky and unsuitable for mass vaccination. The attenuated Senegal isolate described by Jongejan (1991) has not replaced the infection and treatment method because of a lack of crossprotection with most of the *Cowdria* stocks tested so far (Jongejan et al., 1993). More recently, successful protection has been achieved in goats (Martinez et al., 1994), sheep (Mahan et al., 1995) and cattle (Totté et al., 1997) with inactivated *Cowdria* emulsified in Freund's adjuvant.

Research is now focused on the identification of protective antigens which can be produced by recombinant DNA technology. The understanding of immune mechanisms involved in protection and pathology is important in this identification of antigens that will trigger the appropriate response. Studies in mice have shown that the immunity to *Cowdria* infection is mainly T cell mediated. Athymic nude mice were unable to recover from and mount an immune response against *Cowdria* infection (Du Plessis et al., 1991). Protection of naive mice against a virulent challenge was achieved by adoptive transfer of spleen cells from syngenic mice immunised by infection and treatment, and this protection was shown to be confered by the $CD8^+$ fraction of T lymphocytes (Du Plessis, 1982, Du Plessis et al., 1991). $CD4^+$ lymphocytes were unable to transfer resistance. It is generally assumed that a $CD8^+$ mediated response is developed against microorganisms living and multiplying in host cells. Such a response was therefore expected for *Cowdria* infection (Ojcius et al., 1996). However, despite reports that inactivated organisms can be processed for presentation to $CD8^+$ in association with MHC class I molecules (Pfeifer et al., 1993), according to current theories, exogenous antigens like inactivated *Cowdria* are processed through an MHC class II pathway to be presented to $CD4^+$ T cells. Indeed, a helper T lymphocyte response was induced in cattle immunised against heartwater by vaccination with inactivated *Cowdria* in Freund's adjuvant (Totté et al., 1997). *In vitro* stimulation of lymphocytes collected from these immune cattle, induced the proliferation of a $CD4^+$, MHC class II-restricted population of T cells producing interferon γ, which is known to inhibit the growth of *Cowdria in vitro* (Totté et al., 1996).

Recently, we have shown that Montanide ISA 50, a licenced oil adjuvant can efficiently replace Freund's adjuvant for the immunisation of susceptible goats against heartwater with inactivated *Cowdria*. Therefore it will be used in a future first generation vaccine (Martinez et al., 1996). At this stage, it appeared important to further characterize the immune response induced *in vivo* in goats and cattle by inactivated preparations of *Cowdria*, and to compare the effect of both Freund's and ISA 50 adjuvants.
In this study we have analysed by flow cytometry the differentiation of circulating immunocompetent cells of cattle and goats after vaccination with inactivated *Cowdria ruminantium* in Freund's or ISA 50 adjuvant, and after challenge with virulent organisms.

Material and methods

Preparation of Cowdria ruminantium

C. ruminantium (Gardel stock, Uilenberg et al., 1985) was grown in bovine umbilical endothelial cells (BUEC) for the inoculation to cattle, and in caprine endothelial cells for immunisation of goats, by conventional methods (Martinez et al., 1990). Antigens for vaccination were produced as described previously (Martinez et al., 1994). Briefly, when the culture showed between 70-80% cytopathic effect, the supernatant was collected and cell debris was removed by centrifugation at 1000 g for 10 min. The supernatant was further centrifuged at 30,000 g for 30 min and the pellet was resuspended in 0.1M PBS, pH 7.1. *Cowdria* particles were lysed by 5 cycles of freezing in liquid nitrogen to immunise cattle. In the experiments on goats, *Cowdria* was inactivated overnight at 4°C in 0.1% sodium azide without lysis. The resulting preparations were not infective for endothelial cells (data not shown) suggesting that they did not contain viable organisms. Passages 83 and 100 of the Gardel stock were used for the antigen preparation while for the challenge, the virulent passage 45 was used in cattle and the virulent passages 30 and 37 were used in goats.

Immunisation and challenge of animals

Goats

The immunisation and challenge procedures have been described in detail elsewhere (Martinez et al., 1996). Briefly, 32 susceptible goats from Les Saintes (French West Indies) were immunised by two subcutaneous injections at 81 days interval as follow : 5 goats with *Cowdria* in Freund's adjuvant (first and second injection in complete and incomplete Freund's adjuvants respectively, FA+AG group), 5 goats with

Freund's adjuvant as before but with PBS instead of antigen (FA+PBS group), 17 goats with *Cowdria* in Montanide ISA 50 adjuvant (ISA+AG group), 5 goats with PBS in ISA 50 (ISA+PBS group). Naive goats were kept as controls. Animals were challenged at three occasions.
The first challenge administered 3.5 months after vaccination was aimed at comparing the efficacy of Freund's and ISA 50 adjuvants. All goats of the FA+PBS, FA+AG, ISA+PBS groups, and 5 goats from the ISA+AG group were inoculated intravenously together with 5 naive control animals, with 2 ml of untitrated infective culture supernatant. The second and third challenges were administered 15 and 17 months after vaccination respectively, to evaluate the duration of the protection and the effect of the challenge dose on the survival of animals. A dose of 50 tissue culture lethal dose 50% (TCLD50) per goat was given at challenge 2 and a dose of 10 TCLD50 per goat at challenge 3, after *in vitro* titration of the inoculum as described by Totté at al. (1993).

Cattle

Eight 8 month old Ayrshire cattle were obtained from a ranch that practices regular spraying of animals with acáricides. The sera of all animals were negative for antibodies to *Cowdria* as determined by an IFA test (Martinez et al., 1990). Five animals were immunised by intramuscular inoculation of 0.5 ml of *C. ruminantium* lysate formulated in an equal volume of complete Freund's adjuvant. One month later, the calves received a similar inoculation in incomplete Freund's adjuvant. Each animal received a total of approximately 100 µg of *Cowdria* lysate. Two and 3 of the 5 immunised calves were challenged intravenously 3 and 10 months later respectively, with 3 ml of a virulent *Cowdria* preparation derived from *in vitro* cultures. This dose has been schown previously to cause 100% mortality in naive cattle and corresponded to 135 TCLD50. Two animals in the challenge after 3 months and 1 animals in the challenge after 10 months were used as non-immunised control. Previous experiments have schown that neither Freund's adjuvant nor inactivated *Cowdria* alone confer protection against virulent challenge with the agent (Mahan et al,. 1995; Martinez et al., 1996).

Analysis of PBM by flow cytometry

Peripheral blood mononuclear leukocytes (PBMC) were separated by flotation of blood collected in Alsever's solution on Ficoll/Paque (Pharmacia, Uppsala, Sweden). Their surface phenotypes were analysed by indirect immunofluorescence staining using cell-type specific monoclonal antibodies (Mab). Mabs IL-A12 (IgG2a), IL-A51 (IgG1) and GB21A (IgG2b) recognize bovine CD4, CD8 and G/D cell receptor respectively (Baldwin et al., 1986; MacHugh et al., 1991; MacHugh, personal communication). Goat CD4, CD8 and GD cells were analysed using Mabs GC50A1

(IgM), IL-A51 and CC15 (IgG2a) respectively (Bensaid and Hadam, 1991; Morrison and Davis, 1991). Mab IL-A30 (IgG1) recognises bovine cell surface IgM and thus defines B cells (Williams et al., 1990). The population of B cells in goats was analysed with Mab SDU2-104 (Mukwedeya et al., 1996). Mab IL-A24 (IgG1) recognises an uncharacterized heterodimeric molecule on bovine monocytes and polymorphonuclear cells (Ellis et al., 1988) and also recognises goat monocytes. Mab IL-A43 (IgG2a) and Mab MMIA (IgG1) recognise bovine CD2 and CD3 respectively (Davis et al., 1991; 1993). Mab IL-A105 (IgG2a) which recognises the alpha chain of bovine CD8 marker was used for double staining studies (MacHugh et al., 1993). The cells were analysed for cell size (forward angle light scatter) and fluorescence on a fluorescence-activated cell sorter (FACS II, Becton Dickinson, Sunnyvale, CA, USA). The results from FACS analysis are expressed as % of total PBM. For each cell subset, the absolute number per microliter of blood was calculated using the following formula : % of total PBMC X number of leucocytes per µl of blood / 100. Where the number of leucocytes is the total white blood cell count (Cobas Minos Vet, Rocher, France) without granulocytes since these cells do not float on Ficoll/Paque.

Statistical analysis

Differences in cell populations between days, or between treatment for a given day, were compared by variance analysis using SAS program (SAS, 1987). Values obtained from vaccinated goats which survived were compared with values from vaccinated animals which died using Student's *t* test.

Results

No significant change was observed in the composition of immune cell populations after vaccination and boosting. In contrast, very significant changes occured after challenge. The variations observed were very similar whatever the adjuvant used, the time of the challenge and the ruminant species.

Goats

Challenge 1

After the first challenge, all goats in the 3 control groups died, whereas 2 out of 5 animals died in each group vaccinated with *Cowdria* in adjuvant. The changes in sub-populations of PBMC was expressed in relative numbers (% of PBMC). The

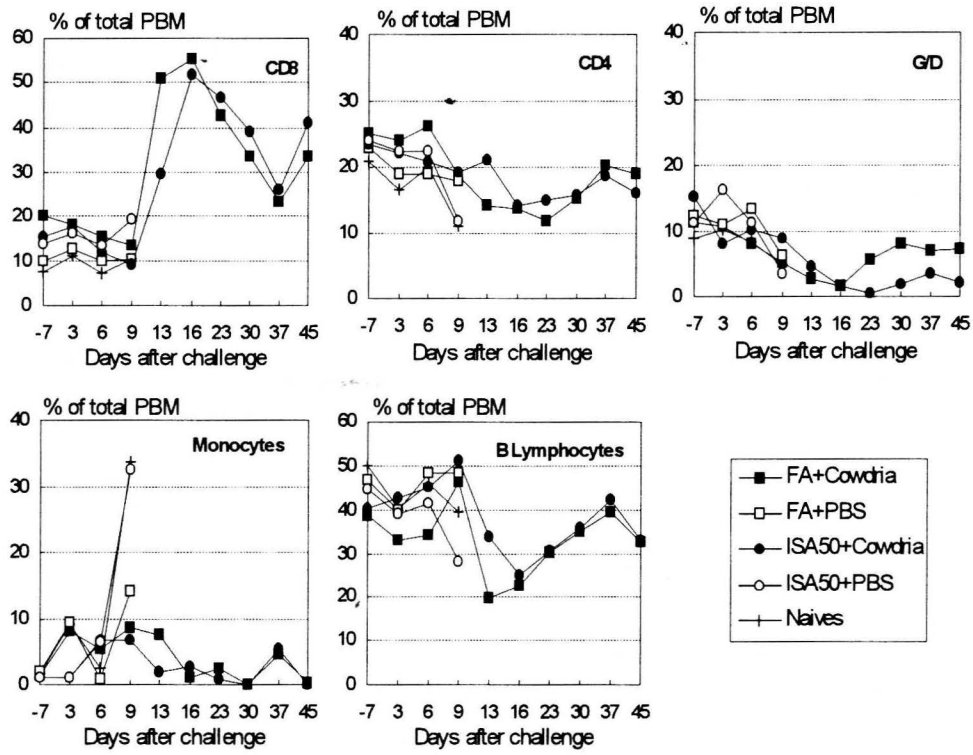


Fig. 1. Evolution of the subsets of peripheral blood monocytes (in percentage of total PBMC) in goats after a virulent challenge administered 3.5 months after vaccination. Each point represents the mean of animals still alive within a group (see text). FA, Freund's adjuvant; ISA50, Montanide ISA 50 adjuvant.

comparison of the effect of the challenge on animals vaccinated with Freund's and ISA 50 adjuvants is presented in Fig. 1.

The percentage of $CD4^+$ T cells in the 3 groups of naive or adjuvant control goats declined either gradually until day 9, or suddenly between days 6 and 9, by which time all the goats died. The drop was statistically significant for the ISA+PBS ($P=0.002$) and FA+PBS ($P=0.05$) groups but not for the control group. In both vaccinated groups, the percentage of $CD4^+$ cells also dropped until a minimum was reached around day 23 post inoculation. $CD4^+$ remained at significant low levels from days 9 to 30 in the FA+AG group ($P=0.02$ to $P<0.001$), and days 16 to 30 in the ISA+AG group ($P=0.05$). However, at day 45, preinoculation levels were not yet recovered.

A 2 to 3 fold drop was also observed in the γδ T cells in all groups. In the control groups, the diminution was significant only for FA+PBS ($P=0.05$) and

FA+AG (P=0.05). As for $CD4^+$ cells, the γδ T cell population continued to decrease after day 9 in the FA+AG group and remained low until day 16 (P=0.05 to P<0.001). For the ISA+AG group, the percentage of γδ T cells decreased significantly only by day 13 and remained at low level until the end of the study at day 45 (P=0.02 to 0.05).

There was no significant change in the percentage of B lymphocytes until day 9. Their level started to decrease at day 13 to compensate a considerable rise of the $CD8^+$ cells which reached more than 50% of the PBMC around day 16. At day 13 all goats vaccinated with Freund's adjuvant had shown a significant rise of $CD8^+$ cells, even in both animals which died at day 14 and 15. The peak appeared slightly later in the group vaccinated with ISA 50. Two out of 5 goats also died in this group at day 11, but without showing an increase of $CD8^+$ cells.

Blood monocytes increased soon after challenge in animals vaccinated with Freund's adjuvant, and remained at significant high levels from day 3 to 13 (P< 0.05). The augmentation occured later (day 6) and persisted for a shorter time (day 9) in goats vaccinated with ISA 50. On 13 out of 24 animals monocytes levels rised above 20%.

Challenge 2 and 3

The variations of blood mononuclear cells after challenges 2 and 3 are expressed in absolute cell numbers and are displayed in Fig. 2. The challenge dose was decreased compared to the first challenge. The dose of 50 TCLD50 per goat inoculated at challenge 2 still proved too high since a mean incubation of 4.5 $\pm$ 1.3 days was observed in the control group. The decrease of the dose to 10 TCLD50 per goat at challenge 3 was closer to a field challenge, since the 4 naive control animals had an incubation of 11 days. All naive animals died after both challenges whereas 3 out of 6 and 5 out of 6 survived in challenge 2 and 3 respectively.

A significant decline in total PBMC count (P=0.05) was observed in all goats during the first 6 to 10 days after challenge 2 after which numbers increased more than 2-fold until day 17. Compared to day 10, the levels of PBMC remained significantly augmented between days 13 and 31. In contrast to challenge 2, no drop was observed after the 3rd challenge. The number of PBMC gradually increased and remained at significant elevated levels from day 13 to 39 (P = 0.01 to P < 0.001). This increase in PBMC was mainly due to a considerable increase of $CD8^+$ cells which remained significantly elevated from day 13 to 31 after challenge 2, and from day 13 to 39 after challenge 3. Although not significant, a 2.2- and a 2.8-fold drop of $CD8^+$ cells was observed at day 10 in challenge 3 and 2, respectively. Monocytes peaked before the $CD8^+$ cells in both challenges. As in challenge 1, the $CD4^+$ and the γδ T cells declined until day 10 in challenge 2 (P = 0.005 and P=0.05 respectively) to return to preinfection levels by day 13 to 17. A drop of γδ and $CD4^+$ cells although non significant, also occured around day 13 after challenge 3. The slight increases of B lymphocytes observed after both challenges was not-significant.

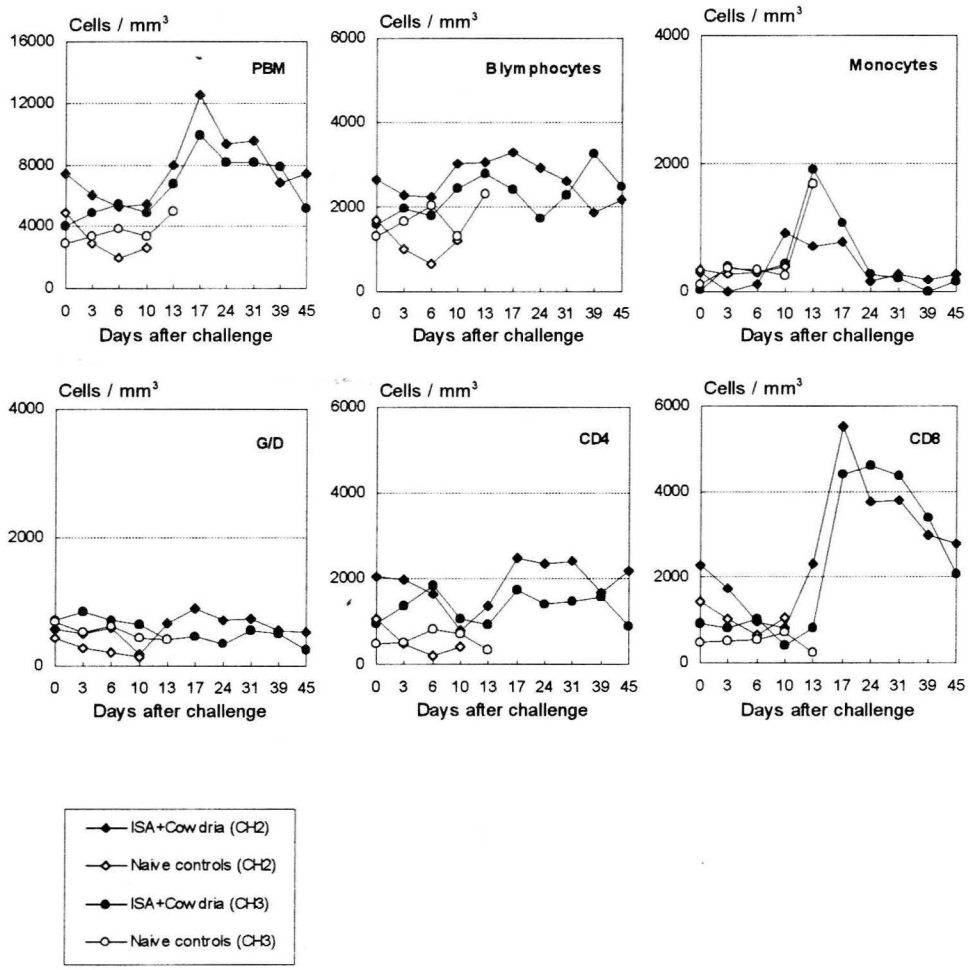


Fig. 2. Changes in the peripheral blood leucocytes (absolute numbers) in goats after virulent challenge 2 (CH2) and challenge 3 (CH3) administered 15 and 17 months after vaccination, respectively. Each point represents the mean of animals still alive within a group (see text).

Due to the low number of animals within groups, the comparison of animals surviving and dying after challenge was possible only after challenge 2 where 3 out of 6 vaccinated goats died and 3 out of 6 survived. The group of 3 animals which survived had a better ability to limit the decrease of lymphocytes observed during the first 10 days after challenge. Thus, at day 10, the surviving group showed an average decrease of 9% of PBMC, 46% of $CD8^+$ cells, 49% of $CD4^+$ cells, 64% of $\gamma\delta$ cells, whereas the decrease was 47% of PBMC, 84% of $CD8^+$ cells, 79% of

$CD4^+$ cells and 84% of γδ cells in the group of animals which died. The decrease of these cell populations was progressive from day 0 to 10. A 35% decrease of B lymphocytes was observed in goats which died, whereas a 63% increased occured in surviving goats (P=0.05). The level of monocytes increased in both groups, and was higher (not significant) in the group of goats which died.

Cattle

All 5 immunised animals survived the challenge while the naive control died of heartwater as confirmed by the presence of *Cowdria* in brain capillaries. Immunised animals reacted with high fever sooner than the control animals but were otherwise clinically asymptomatic.

The changes in absolute numbers of the different PBMC populations during challenge shown in Fig. 3 are remarquably similar to that observed in goats. A gradual decline in PBMC, $CD4^+$ cells, B lymphocyte and to a lesser extent $CD8^+$ cell counts was observed in all animals during the first 10 days after challenge. No significant change was observed in the number of γδ T cells. Blood monocytes increased in number soon after challenge in the immunised animals whereas in the control calves the rise in monocyte numbers occured late, just before death. Considerable increase in the numbers of circulating $CD8^+$ lymphocytes was observed in all immunised animals from 2 weeks on after challenge. At day 19 post-challenge, the $CD8^+$ cell population represented 40 to 55% of total PBMC compared to only 15 to 20% before challenge. FACS analysis (double staining) showed that the majority of these cells expressed CD3 but not the γδ TCR, suggesting that they were αβ T cells and not γδ T cells nor NK-like cells. There was also a significant (2.5- to 3-fold) increase in the percentage of $CD8^+$ blasts with a peak around day 17 after challenge (data not shown).

Discussion

The present study was designed to characterize by flow cytometry the shift between subpopulations of immunocompetent cells in the circulation of goats and cattle immunised against heartwater with inactivated *Cowdria* emulsified in an oil adjuvant, and submitted to a lethal challenge at different times after vaccination.

Significant changes were observed only after challenges. A decrease of $CD4^+$, $CD8^+$ and γδ T cell subsets occured progressively during the first 10 days after challenge in cattle, and in the first two challenges in goats. In contrast, this diminution was not noticed after the third challenge of goats. In the absence of significant changes in B cell numbers, the lymphopenia (from 7233 to 4023 cell μl^{-1} between day 0 and day 10, data not shown) observed after challenge 2 can be

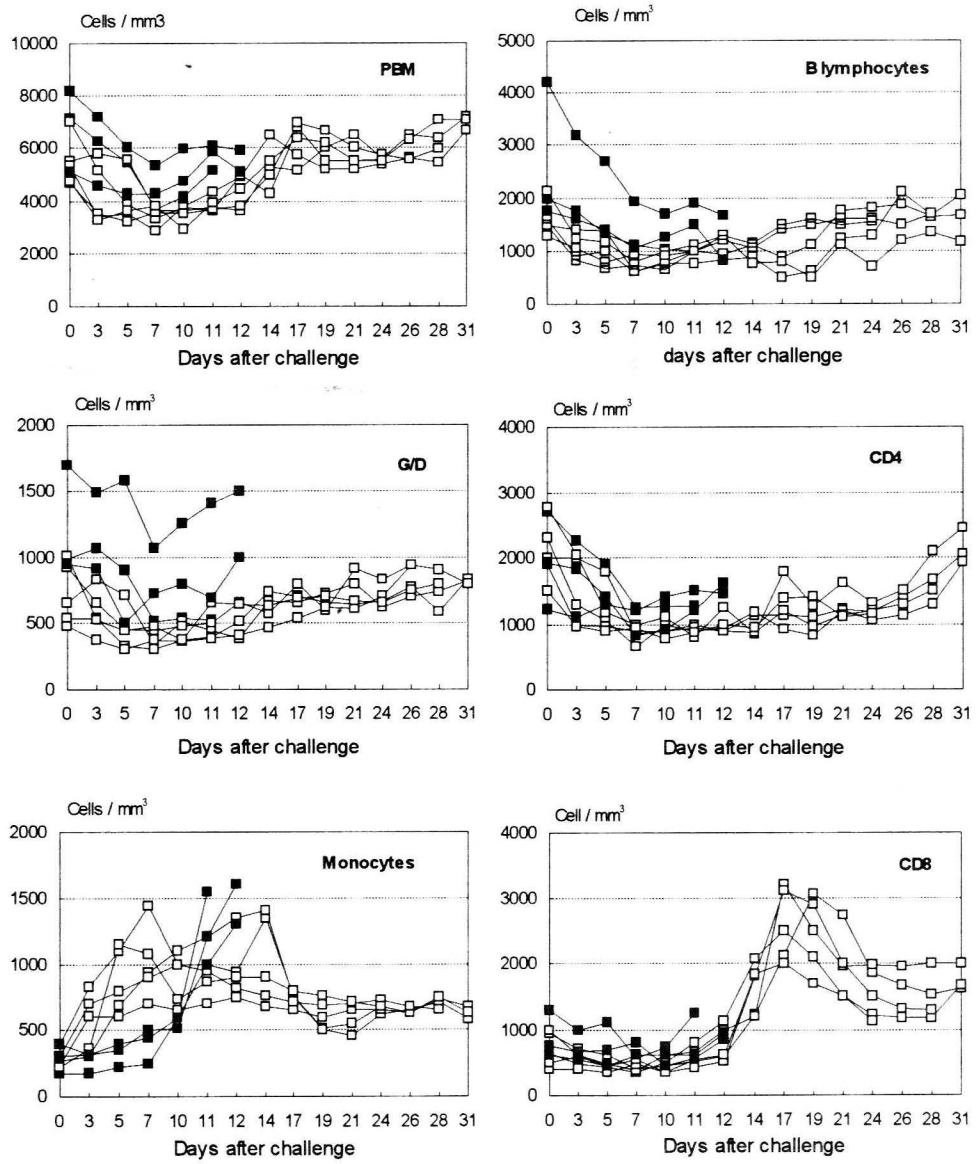


Fig. 3.
Changes in peripheral blood leucocytes (in absolute numbers) in cattle after a virulent challenge administered 3 months (2 animals) and 10 months (3 animals) after vaccination with inactivated Cowdria in Freund's adjuvant. The curves of all animals are represented to illustrate the variability between individuals. (■) control animals, (□) vaccinated animals.

attributed to a selective drop of T lymphocytes. No lymphopenia was observed in challenge 3. The dose of the virulent inoculum may be responsible for this difference since all challenges except challenge 3 were done with highly virulent inocula as illustrated by the very short incubation periods obtained. This dose effect may account for conflicting results between authors who reported either a leucopenia mainly due to a lymphopenia (Ilemobade and Blotkamp, 1978), or a lymphocytosis (Van Amstel et al., 1987). A depletion of $CD4^+$ lymphocytes from 41% to 24% of PBMC was also observed in mice after inoculation with *Cowdria* (Du Plessis et al., 1992). Interestingly, adoptive transfer experiments showed that spleen $CD4^+$ T cell were unable to confer protection to challenge in recipient mice (Du Plessis et al., 1991). An important lymphocytopenia due to a significant decrease of all T cell subsets as well as B lymphocytes in peripheral blood of sheep, was also reported during the period of parasitaemia in the first days following infection by *Ehrlichia phagocytophila* (Woldehiwet, 1991), a pathogen related to *Cowdria* (Van Vliet et al., 1992). Infection was associated with an impaired function of infected neutrophils and monocytes (Woldehiwet, 1987a) and to a reduced responsiveness of lymphocytes to lectins (Woldehiwet, 1987b).

In the present study, the decrease of the $CD4^+$ cell number and the absence of blasts in this cell population, suggest that their role was limited although it has been shown previously that cattle immunised with lysed *Cowdria* in CFA are capable of eliciting a *Cowdria* specific $CD4^+$ T cell response *in vitro* (Totté et al., 1997). However, it was observed that goats which survived after challenge 2, showed a better ability than goats which died to limit the decrease of their lymphocyte populations, and preliminary studies have shown that PBMC collected during the phase of depletion can proliferate *in vitro* after stimulation with *Cowdria* lysates (Totté, unpublished data). Whether this stimulation is lowered compared to a prechallenge stimulation, or is related to survival needs further studies

The lymphopenia was not paralleled by a neutropenia (data not shown), in opposition to the reports of Ilemobade and Blotkamp (1978), Abdel Rahim and Shommein (1977) and Van Amstel et al. (1987). In contrast, an important rise of monocytes occured within 2 weeks after challenge. The earliness of this increase was not related to the survival rate in goats. This increase was observed earlier with Freund's adjuvant than with ISA 50 in the first challenge of goats, but the same number of animals survived. Moreover, the increase of blood monocytes occured slightly later in challenge 3 where 5 out of 6 goats survived, than in challenge 2 where 3 out of 6 goats survived. In contrast, the 5 immunised cattle which survived had a very early increase compared with control animals in which the increase in monocyte numbers only occured shortly before death. These discrepancies suggest either differences between both species, or that the evolution of the disease is related to the activity of these monocytes rather than to their number. Indeed, whatever the group of animals and the course of the disease, a characteristic feature at microscopic examination of blood smears performed in challenge 2 and 3 on goats, was the activation of monocytes resembling macrophages with enlarged size,

polylobulated nuclei and vacuolated cytoplasm. This aspect of macrophages was present in the 20 goats examined, even those which did not show a significant increase of the monocyte numbers. The presence of activated monocytes has also been regularly observed in field cases according to Van Amstel et al. (1987). Macrophages are known to play a pivotal role in early protection against intracellular bacterial infections (Cheers and Zhan, 1996), particularly as potent producers of IL-12 which acts as an inducer of interferon γ by NK and T cells, and favors differentiation of T cells towards a Th1 phenotype (Kaufmann, 1995; Trinchieri, 1995). Functional analysis of macrophages early after infection by Cowdria has to be conducted.

The main pathological feature however, was the considerable increase of $CD8^+$ T cells in all animals (controls or immunised) which lasted at least until day 13 after challenge. The adjuvant used, the dose of the challenge and the ruminant species had little effect on the occurrence of this peak which reached a maximum around days 16 to 19. The number of $CD8^+$ blasts increased strongly during the peak and most of them had a conventional $\alpha\beta$ T cell receptor. Du Plessis et al. (1992) also showed that percentages of $CD8^+$ cells in immune mice increased more than 3 fold after a reinfection with *Cowdria*, and remained at high levels at least until day 112. These $CD8^+$ cells were able to confer resistance to cowdriosis in recipient mice after adoptive transfer (Du Plessis et al., 1991). The functional characterisation of these cells in ruminants is underway. Although these results suggest a possible role for $CD8^+$ T lymphocytes in the resistance to cowdriosis, it should be pointed out that they did not increase in number until 2 weeks after challenge, by which time the control animal had died. Other mechanisms of protection may therefore take place before a $CD8^+$-mediated response is generated.

In conclusion, this study revealed three main pathological features after inoculation of ruminants with virulent *Cowdria* : a progressive depletion of T cell subsets during the first 10 days leading to a lymphopenia, a rise and a strong activation of monocytes, and finally a considerable rise of $CD8^+$ T lymphocytes. However, the changes in immune cell populations were studied in the blood circulation only. We cannot exclude a situation where immune responses are taking place locally, e.g. in the lymph nodes and/or the spleen, and are not accurately represented in the blood. As a matter of fact the presence of *Cowdria* has been described in lymph nodes of sheep infected by *Cowdria* as early as 2 to 4 days after infection (Du Plessis, 1970). A study of T cell responses to *Cowdria* in other immune compartments of the host is underway. In addition, cell transfer experiments from immune animals to naive twin recipients using different purified lymphocyte populations are also being considered.

Acknowledgements

This work was supported by a grant from the European Community under contract N° TS3-CT91-0007.

References

1. Abdel Rahim, A.I., Shommein, A.M., 1977. Haematological studies in goats experimentally infected with *Rickettsia ruminantium*. *Bull. Anim. Hlth. Prod. Africa*, 26, 232-235.

2. Baldwin, C.L., Teale, A.J., Naessens, J.G., Goddeeris, B.M., MacHugh, N.D., Morrison, W.I., 1986. Characterization of a subset of bovine T lymphocytes that express BoT4 by monoclonals and function : similarity to lymphocytes defined by human T4 and murine L3T4. *J. Immunol.* 136, 4385-4391.

3. Bensaid, A., Hadam, M., 1991. Bovine CD4 (BoCD4). *Vet. Immunol. Immunopathol.* 27, 51-54.

4. Cheers, C., Zhan, Y., 1996. How do macrophages distinguish the living from the dead. *Trends Microbiol.* 11, 453-455.

5. Davis, W.C., Splitter, G.S., 1991. Bovine CD2 (BoCD2). *Vet. Immunol. Immunopathol.* 27, 43-50.

6. Davis, W.C., MacHugh, N.D., Park, Y.H., Hamilton, M.J., Wyatt, C.R., 1993. Identification of a monoclonal antibody reactive with the bovine ortholog of CD3 (BoCD3).). *Vet. Immunol. Immunopathol.* 39, 85-91.

7. Du Plessis, J.L., 1970. Pathogenesis of heartwater : I. *Cowdria ruminantium* in the lymph nodes of domestic ruminants. *Onderstepoort J. Vet. Res.* 37, 89-96.

8. Du Plessis, J.L., 1982. Mice infected with a *Cowdria ruminantium*-like agent as a model in the study of heartwater. Thesis, Veterinary Science, University of Pretoria, South Africa.

9. Du Plessis, J.L., Berche, P., Van Gas, L., 1991. T cell-mediated immunity to *Cowdria ruminantium* in mice : the protective role of $Lyt2^+$ T cells. *Onderstepoort J. Vet. Res.* 58, 171-179.

10. Du Plessis, J.L., Gray, P., C., Van Strijp, F., 1992. Flow cytometric analysis of T cell response in mice infected with *Cowdria ruminantium*. *Onderstepoort J. Vet. Res.* 59, 337-338.

11. Ellis, J.A., Davis, W.C., MacHugh, N.D., Emery, D.L., Kaushal, A., Morrison, W.I., 1988. Differentiation antigens on bovine mononuclear phagocytes identified by monoclonal antibodies. *Vet. Immunol. Immunopathol.* 19, 325-340.

12. Ilemobade, A.A., Blotkamp, C., 1978. Clinico-pathological study on heartwater in goats. *J. Trop. Med. Parasitol.* 29, 71-76.

13. Jongejan, F., 1991. Protective immunity to hearwater *(Cowdria ruminantium* infection) is acquired after vaccination with *in vitro*-attenuated *Rickettsiae*. *Infect. Immun.* 59, 729-731.

14. Jongejan, F., Vogel, S., Gueye, A., Uilenberg, G., 1993. Vaccination against heartwater using *in vitro* attenuated *Cowdria ruminantium* organisms. *Rev. Elev. Med. vet. Pays trop.* 46, 223-227.

15. Kaufmann, S.H.E., 1995. Immunity to intracellular microbial pathogens. *Immunol. Today* 16, 338-342.

16. Mac Hugh, N.D., Bensaid, A., Howards, C.J., Davis, W.C., Morrison, W.I., 1991. Analysis of the reactivity of anti-bovine CD8 monoclonal antibodies with cloned T cell lines and mouse L-cells transfected with bovine CD8. *Vet. Immunol. Immunopathol.* 27, 169-172.

17. Mac Hugh, N.D., Taracha, E.L., Toye, P.G., 1993. Reactivity of workshop antibodies on L cells and COS cell transfectants bovine CD antigens. *Vet. Immunol. Immunopathol.* 39, 61-67.

18. Mahan, S.M., Andrew, H.R., Tebele, N., Burridge, M.J., Barbet, A.F. 1995. Immunisation of sheep against heartwater with inactivated *Cowdria ruminantium*. *Res. Vet. Sci.* 58, 46-49.

19. Martinez, D., Swinkels, J., Camus, E., & Jongejan, F. 1990. Comparaison de trois antigènes pour le diagnostic de la cowdriose par immunofluorescence indirecte. *Rev. Elev. Méd. vét. Pays trop.* 43, 159-166.

20. Martinez, D., Maillard, J.C., Coisne, S., Sheikboudou, C., Bensaid, A., 1994. Protection of goats against heartwater acquired by immunisation with inactivated elementary bodies of *Cowdria ruminantium*. *Vet. Immunol. Immunopathol.* 41, 153-163.

21. Martinez, D., Perez, J.M., Sheikboudou, C., Debus, A., Bensaid, A., 1996. Comparative efficacy of Freund's and Montanide ISA50 adjuvants for the immunisation of goats against heartwater with inactivated *Cowdria ruminantium*. *Vet. Parasitol.* 67, 175-184.

22. Morrison, W.I., Davis, W.C., 1991. Differentiation antigens expressed predominantly on CD4- CD8- T lymphocytes (WC1, WC2). *Vet. Immunol. Immunopathol.* 27, 71-76.

23. Mukwedeya, D.T., Takamatsu, H., Denyer, M.S., Parkhouse, R.M.E., 1996. Analysis of bovine B cells reactive monoclonal antobodies. *Vet. Immunol. Immunopathol.* 52, 285-294.

24. Neitz, W.O., Alexander, R.A., 1945. Immunisation of cattle against heartwater and the control of the tick-borne diseases, redwater, gallsickness and heartwater. *Onderstepoort J. Vet. Sci. Ani. Ind.* 20, 137-152.

25. Ojcius, D.M., Gachelin, G., Dautry-Varsat, A., 1996. Presentation of antigens derived from microorganisms residing in host-cell vacuoles. *Trends Microbiol.* 4, 53-59.

26. Pfeifer, J.D., Wick, M.J., Roberts, R.L., Findlay, K., Normark, S.J., Harding, C.V., 1993. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature* 361, 359-362.

27. Provost, A., Bezuidenhout, J.D., 1987. The historical background and global importance of heartwater. *Onderstepoort J. vet. Res.* 54, 165-169.

28. SAS Institute Inc., 1987. Guide for personnal computers, Version 6 Edition., NC: SAS Institute Inc. USA.

29. Totté, P., Blankaert, D., Zilimwabagabo, P., Werenne, J., 1993. Inhibition of *Cowdria ruminantium* yield by interferons alpha and gamma in endothelial cells. *Rev. Elev. Méd. Vét. Pays trop.* 46, 189-194.

30. Totté, P., Vachiery, N., Martinez D., Trap, I., Ballingdall, K.T., MacHugh, N.D., Bensaid, A., Werenne, J., 1996. Recombinant bovine interferon gamma inhibits the growth of *Cowdria ruminantium* but fails to induce major histocompatibility complex class II following infection of endothelial cells. *Vet. Immunol. Immunopathol.* 53, 61-71.

31. Totté, P., MacKeever, D., Martinez, D., Bensaid, A., 1997. Protection of cattle against cowdriosis by vaccination with inactivated elementary bodies of *Cowdria ruminantium*. *Infect. Immun.* 65, 236-241.

32. Trinchieri, G., 1995. Interleukin-12 : a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Ann. Rev. Immunol.* 13, 251-276.

33. Uilenberg, G., 1983. Heartwater (*Cowdria ruminantium* infection) : current status. *Adv. vet. Sci. Comp. Med.* 27, 427-480.

34. Uilenberg, G., Camus, E., Barré, N., 1985. Quelques observations sur une souche de *Cowdria ruminantium* isolée en Guadeloupe (Antilles Françaises). *Rev. Elev. Méd. Vét. Pays trop.* 38, 34-42.

35. Uilenberg, G., 1995. International collaborative research : significance of tick-borne hemoparasitic diseases to world animal health. *Vet. Parasitol.* 57, 19-41.

36. Van Amstel, S.R., Guthrié, A.J., Reyers, F., Bertschinger, H., Oberem, P.T., Killeen, V.M., Matthee, O., 1987. The clinical pathology and pathophysiology of heartwater : a review. *Onderstepoort J. Vet. Res.* 54, 287-290.

37. Van Vliet, A. H. M., F. Jongejan, and B. A. M. van der Zeijst. 1992. Phylogenetic position of *Cowdria ruminantium* (*Rickettsiales*) determined by analysis of amplified 16S ribosomal DNA seqences. *Int. J. Syst. Bact.* 42, 494-498.

38. Williams, D.J.L., Newson, J., Naessens, J., 1990. Quantitation of bovine immunoglobulin isotypes and allotypes using monoclonal antibodies. *Vet. Immunol. Immunopathol.* 24, 267-283.

39. Woldehiwet, Z., 1987a. The effects of tick-borne fever on some functions of polymorphonuclear cells of sheep. *J. Comp. Pathol.* 97, 481-485.

40. Woldehiwet, Z., 1987b. Depression of lymphocyte response to mitogens in sheep infected with tick-borne fever. *J. Comp. Pathol.* 97, 637-643.

41. Woldehiwet, Z., 1991. Lymphocyte subpopulations in peripheral blood of sheep experimentally infected with tick-borne fever. *Res. Vet. Sci.* 51, 40-43.

CHAPTER 9

Detection of genomic polymorphisms among isolates of the intracellular bacterium *Cowdria ruminantium* by random amplified polymorphic DNA and Southern blotting

J.M. Perez [a], D. Martinez [a], A. Debus [a], C. Sheikboudou [a], A. Bensaid [b]

[a] *Centre International en Recherche Agronomique pour le Développement, Département d'Elevage et de Médecine Vétérinaire des Pays Tropicaux (CIRAD-EMVT), BP 1232, 97184 Pointe à Pitre Cedex, Guadeloupe, West Indies.*

[b] *CIRAD-EMVT, BP 5035, 34032 Montpellier, France.*

Submitted

Abstract

Sixteen primers were successfully used in an RAPD assay to generate reproducible fingerprints for 6 isolates (stocks) of *Cowdria ruminantium*, a tick transmitted rickettsia of ruminants. Distinction among stocks was possible by using 1 or at the most 2 primers. Two stocks were very similar although originating from widely distant geographical regions. A genetic distance tree was constructed by analysing 108 fragments in pairwise comparison between stocks. Three amplification fragments probed with *C. ruminantium* genomic DNA determined a restriction fragment length polymorphism which allowed the distinction among stocks except for the two stocks that had similar RAPD patterns. The potential of RAPD to determine the extent of the genetic diversity of *C. ruminantium*, and to develop probes or PCR primers for diagnostic is discussed.

Introduction

The tick-borne rickettsia *Cowdria ruminantium* is the causative agent of cowdriosis which affects wild and domestic ruminants in sub-Saharan Africa and several Caribbean islands (Provost and Bezuidenhout, 1987). It is an obligate parasite of endothelial cells with a tropism for brain microvasculature. Up to now, protection of livestock was achieved by control of the vectors (Jongejan and Uilenberg, 1994) or immunisation by an infection and treatment procedure (Bezuidenhout, 1989). However, immunisation with inactivated bacteria formulated in oil adjuvants was reported recently for goats (Martinez et al., 1994) and sheep (Mahan et al., 1995). Research is being conducted to identify relevant antigens which can be incorporated into recombinant vaccines. However, besides immunological studies, there is a need to evaluate the extent of the genetic and antigenic diversity of this organism. Indeed, the level of cross-immunity induced by different isolates (stocks) is variable (Jongejan et al., 1991). However, no efficient method has yet been described to type the isolates. Serology has not led to the identification of true serotypes (Martinez et al., 1990). Few highly conserved genes have been cloned (Van Vliet et al., 1994; Mahan et al., 1994; Lally et al., 1995). The study of their polymorphism is rather limited (Reddy et al., 1996) and the results are unlikely to correlate with the *C. ruminantium* cross-immunity profile. The PCR technology, using arbitrary primers, has been successfully used for typing strains or isolates of a wide range of microorganisms. It is able to detect polymorphisms widely dispersed in the genome without requiring any prior DNA sequence information (Welsh and McClelland, 1990; Williams et al., 1990), and it therefore appeared to be suitable for application on *Cowdria*.

In this study, we describe the use of the RAPD assay to differentiate isolates of *C. ruminantium* and to examine the extent of its genetic diversity, also with the aim to develop probes for diagnosis in tick vectors and in infected animals.

Material and methods

C. ruminantium isolates.

Six stocks of *C. ruminantium* from widely distant areas were used: one from Guadeloupe in the Caribbean (Gardel stock) (Uilenberg et al., 1985), one from West Africa (Senegal stock at passage 4 in culture, not attenuated) (Jongejan et al., 1988), one from Central Africa (Cameroun stock), two from East Africa (Umpala stock from Mozambique and Lutale stock from Zambia (Jongejan et al., 1988) and one from South Africa (Welgevonden stock) (Du Plessis, 1985).

C. ruminantium cultivation and DNA extraction.

C. ruminantium was cultured in bovine umbilical endothelial cells (BUEC) by conventional methods (Martinez et al., 1990). Elementary bodies (EB) were purified from culture supernatant by differential centrifugations (Martinez et al., 1994) and resuspended in 350 µl of PBS (130 mM NaCl, 4 mM Na_2HPO_4, 1.5 mM KH_2PO_4) to which was added 150 µl of buffer containing 25 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$ and 125 µg of DNAse in order to remove contaminating host cell DNA (Cox et al., 1988). After 90 min of incubation at 37°C, the reaction was stopped by adding 25 mM EDTA. The EB were washed three times in water and lysed by an overnight incubation at 55°C in a solution of 100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 25 mM EDTA, 1.5% SDS, and 250 µg ml^{-1} of proteinase K. Bacterial DNA was then extracted with phenol-chloroform, precipitated with cold ethanol and redissolved in water. To determine the level of contamination with cell DNA, 2 dilutions of bovine DNA (12.5 and 25 ng) and of each preparation of *Cowdria* DNA (500 and 250 ng) were spotted on a Nylon membrane using a slot blot filtration manifold (Hoefer Scientific Instruments) and hybridized with 50 ng ml^{-1} of bovine DNA labelled with digoxigenin as described below.

PCR amplification

Thirty-six decamer arbitrary primers, and primers AB128 (20 nucleotides) and AB129 (25 nucleotides) used for diagnosis (Mahan et al., 1992) were used to randomly amplify *C. ruminantium* genomic DNA. The amplification reaction was performed in a volume of 25 µl containing 1.25 µl 20X polymerase buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP (New England Biolabs), 0.5 U *Tfl* DNA polymerase (Epicentre Technology), 0.2 µM of primer and 25 ng of template DNA. Following an initial denaturation of 6 min at 94°C, the PCR was carried out for 45 cycles in a Perkin Elmer Thermal Cycler (Norwalk, Conn) programmed for 1 min at 94°C, 1 min at 36°C and 2 min at 72°C, followed by a final chain elongation for 10 min at 72°C. Amplified products were electrophoresed in 1.5 % agarose gels containing 0.5 µl ml^{-1} of ethidium bromide. Gels were photographed under UV light. A minimum of two assays was performed for each primer on two different DNA batches of each *C. ruminantium* isolate. Genomic DNA of BUEC was used as a negative control.

Probe preparation, Southern blotting and hybridization

Genomic DNA (1.5 µg) of the six stocks of *C. ruminantium* and BUEC DNA (3 µg), were digested by *Rsa*I. The restriction products were electrophoresed in a 0.8 % agarose gel and blotted onto Nylon N+ membranes (Boehringer) by capillary transfer (Sambrook et al., 1989). Amplified DNA fragments of interest separated in

low melting point agarose gels were excised and purified using a WIZARD PCR Prep DNA purification kit (Promega). They were labelled with digoxigenin by using a commercial kit (Boehringer) and used as probes. Membranes were prehybridized overnight at 68°C in 5 X SSC containing 1% (w/v) blocking reagent (Boehringer), 0.1% (w/v) N-lauroylsarcosine, 0.5% (w/v) SDS, and 100 μg ml^{-1} of denatured salmon sperm DNA. Hybridization was performed overnight at 68°C with 30 ng ml^{-1} of probes in prehybridization buffer. The membranes were then washed successively in 2 X SSC/0.5% SDS at room temperature, and in 0.1 X SSC/0.5 % SDS at 68°C. Hybridized probes were detected by luminescence.

Calculation of similarity coefficients

Pairwise comparisons of amplified DNA fragments generated by different primers among isolates were made. Similarity coefficients between each pair of isolates were calculated as described by Sokal and Michener (Sokal and Mitchener, 1958). A pairwise distance was calculated and used to construct a dendrogram using the unweighted pair-group method of arithmetic averages (UPGMA) of the SAS program (SAS Institute Inc.)

Results

Differentiation of isolates

Among 38 arbitrary primers with G+C contents varying from 50% to 80%, 18 gave an informative array of fragments. Sixteen of these, 14 decamers and primers AB128 and AB129, gave polymorphic patterns and were subsequently used for the study. The number of bands revealed by each primer varied from 1 to 13 (Fig. 1). The patterns obtained with different DNA preparations of the same stock were always identical and no major DNA fragment common to BUEC and *C. ruminantium* was amplified. By comparing the intensity of hybridization signals on dots and considering the ratios of homologous and heterologous DNA, the contamination of *Cowdria* DNA preparations by host cell DNA appeared to be far lower than 1% (Fig. 2). Welgevonden and Cameroun stocks differed by only one fragment on the fingerprint generated by primer 31 (Fig. 1), but each isolate was distinguishable by using one, or at most, two primers.

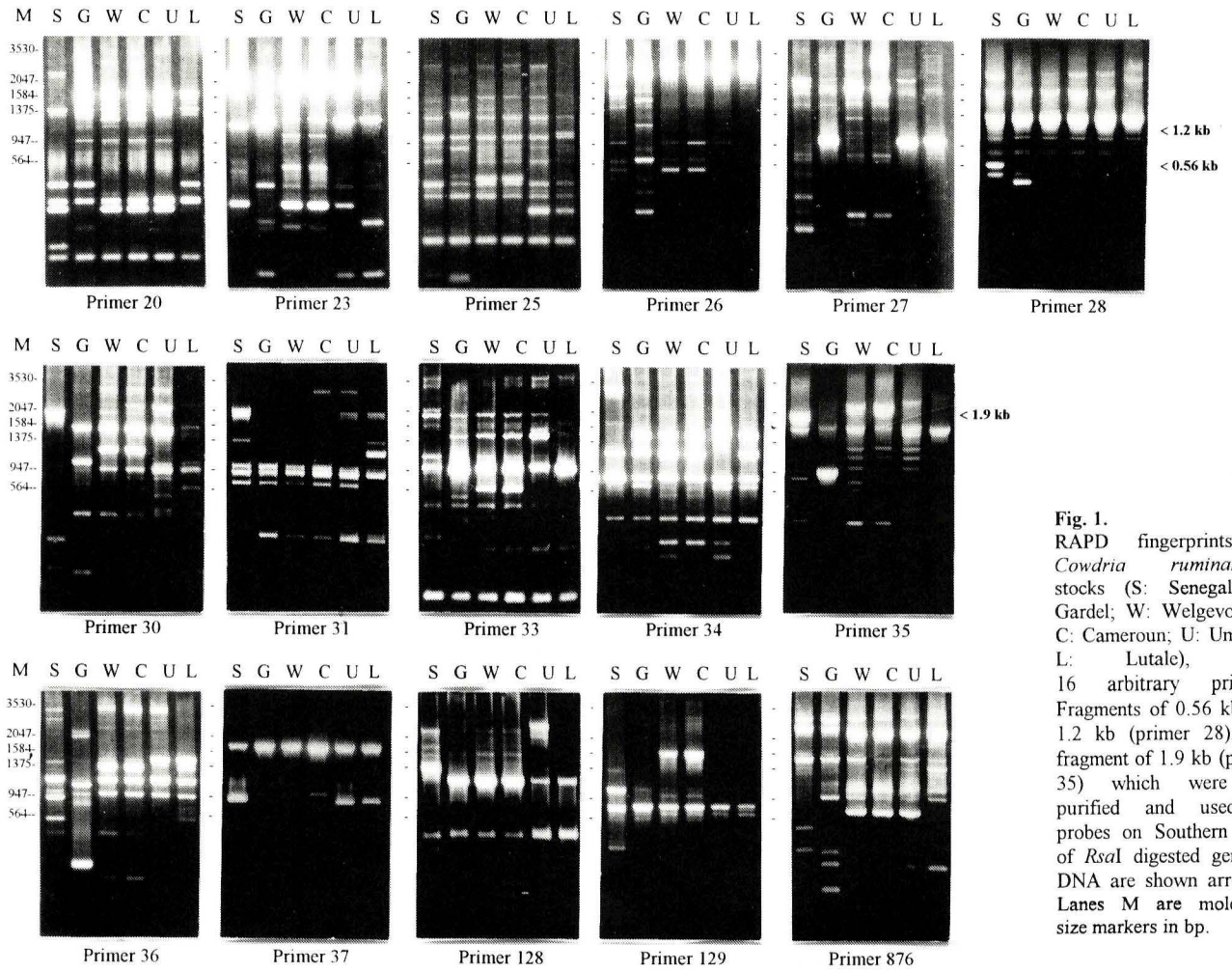


Fig. 1.
RAPD fingerprints of *Cowdria ruminantium* stocks (S: Senegal; G: Gardel; W: Welgevonden; C: Cameroun; U: Umpala; L: Lutale), with 16 arbitrary primers. Fragments of 0.56 kb and 1.2 kb (primer 28), and fragment of 1.9 kb (primer 35) which were gel purified and used as probes on Southern blots of *Rsa*I digested genomic DNA are shown arrowed. Lanes M are molecular size markers in bp.

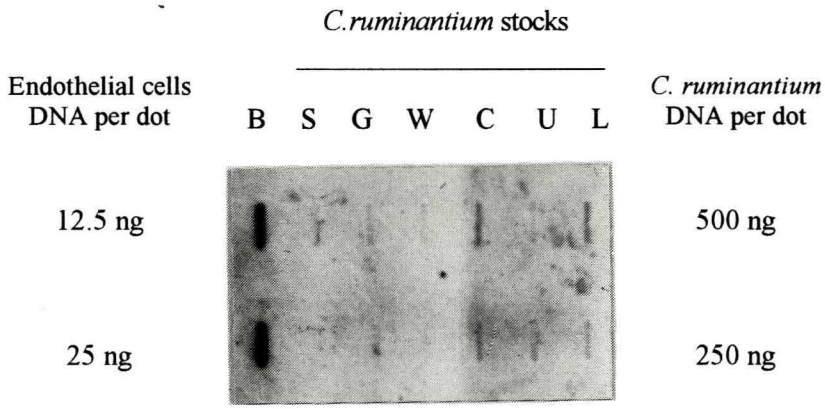


Fig.2.
Hybridization of genomic DNA derived from bovine endothelial cells, with itself (B) and with DNA from 6 stocks of *C. Ruminantium*. Abbreviations of *Cowdria* isolates S, G, W, C, U, L, as in Fig.1.

Genetic distances between isolates

A total of 108 RAPD products generated by the 16 primers was analysed. Minor bands whose intensity varied between amplifications were excluded from the analysis. The resulting dendrogram is shown in Fig. 3. The Senegal stock appears to be most distant from all other stocks. In contrast, Welgevonden and Cameroun isolates were very closely related. Umpala and Lutale were grouped together but did not appear to be closely related. Gardel was located between Senegal and the clusters formed by the other 4 stocks.

Southern blotting and hybridizations.

To identify shared and stock-specific amplification products, three amplification fragments were used as probes in Southern blots to *Rsa*I digested genomic DNA of the six isolates of *C. ruminantium*.

A DNA fragment of 1.2 kb amplified from the 6 stocks was purified from the reaction with primer 28 (Fig. 1). Two probes were derived, one from the Senegal PCR reaction product and one from a mixture of the 1.2 kb fragments of the 6 stocks. After hybridization, the Southern blots showed the same pattern with both

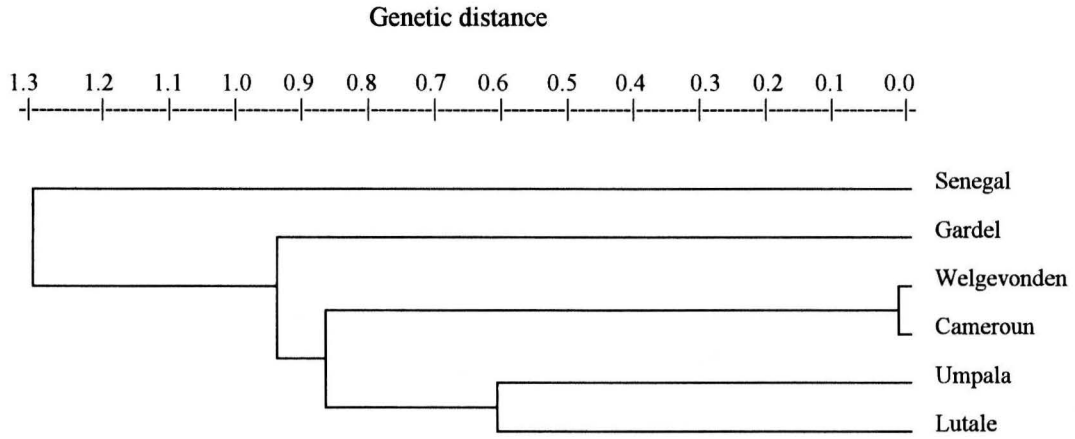


Fig. 3.
Tree of genetic distance between 6 stocks of *C. ruminantium* based on an UPGMA analysis of presence-absence data for RAPD fragments amplified with 16 random primers. Abbreviations of isolates S, G, W, C, U, L, as in Fig. 1. Low values on the genetic distance axis represents high genetic relatedness between stocks and conversely.

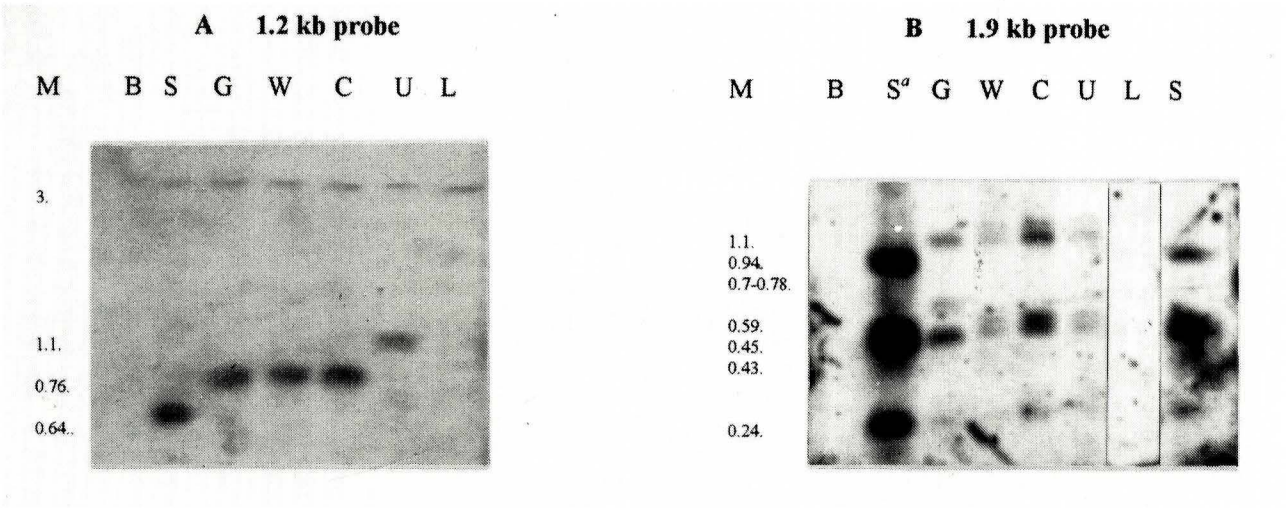


Fig. 4.
Southern blots of *Rsa*I digested genomic DNA of 6 stocks of *C. ruminantium* (1.5 µg of DNA digested per stock except S^a, 15 µg digested) and uninfected BUEC (3 µg of DNA digested) probed with A) RAPD fragment of 1.2 kb amplified by primer 28 and B) RAPD fragment of 1.9 kb amplified by primer 35. Abbreviations of isolates S, G, W, C, U, L, are as in Fig. 1. Lanes M are sizes of fragments which hybridized on the blots.

probes (Fig. 4A). One monomorphic fragment of 3 kb common for all 6 stocks and a polymorphic region were revealed. Genomic polymorphic fragments of 0.64 and 1.1 kb were detected for Senegal and Umpala respectively, while the same 0.76 kb DNA fragment characterized Gardel, Welgevonden and Cameroun stocks. Except for the 3 kb fragment, the probe did not hybridize to the Lutale genomic DNA.

A second probe was derived from a fragment of 0.56 kb appearing specific for the Senegal stock on the pattern obtained with the primer 28 (Fig. 1). The Southern blot pattern was similar to that obtained with the first probe except that the 3 kb fragment could not be revealed (result not shown).

A third probe of 1.9 kb was derived from a fragment obtained with primer 35 which appeared specific for the Senegal stock (Fig. 1). As shown in Fig. 4B, this fragment hybridized to the DNA of all stocks except Lutale. Two DNA fragments of 0.24 kb and 0.45 kb were common to the 5 positive stocks. Welgevonden, Cameroun and Umpala stocks showed the same pattern consisting of 5 bands : the band of 0.24kb, a doublet of 0.45 kb and 0.52 kb, a second doublet of 0.94 kb and 1.1 kb. Two DNA fragments of 0.59 kb and 0.78 kb were specific for the Gardel and the Senegal isolates respectively.

The RFLP profiles obtained on Southern blots made it possible to differentiate *C. ruminantium* isolates. Gardel, Welgevonden and Cameroun stocks were similar on the blot probed with the 1.2 kb fragment (Fig. 4A). However, Gardel could be differentiated from these 2 stocks when the blot was probed with the 1.9 kb fragment (Fig. 4B).On the other hand, Welgevonden, Cameroun and Umpala were similar with the 1.9 kb probe but Umpala exhibited a different profile with the 1.2 kb probe. As in the RAPD analysis, Welgevonden and Cameroun isolates were identical.

Discussion

In this study, the RAPD technique was applied successfully to generate DNA fingerprints which were polymorphic between isolates of the intracellular bacterium *C. ruminantium* (Fig. 1). The technique was reproducible since similar patterns were consistently obtained between independant DNA preparations. Contamination with host cell DNA was very limited (Fig. 2). Moreover, although the cultivation of *C. ruminantium* was always done in the same cell line, other assays have shown that the reproducibility of the pattern was not influenced by the origin of the cells (cattle or goat). Finally, the probes developed from purified RAPD products did not hybridize with host cell DNA used as control on Southern blots (Fig. 4), demonstrating that the fragments were specific for the bacterial genome.

A significant diversity in PCR amplified fragments was demonstrated between isolates of *C. ruminantium* to such a degree that only 24% of the major bands were common among the 6 stocks. Despite the observed diversity, the Welgevonden and Cameroun stocks, although originating from very distant regions

were very similar. Differentiation among the other stocks studied, could be made by using one or the combination of two RAPD fingerprints. Besides a simple differentiation among stocks, analysis of RAPD fingerprints can be used to conduct genetic studies and illustrate the relatedness of isolates (Ralph et al., 1993). A basic asumption in such studies, is that bands of similar size are homologous. This can lead to misinterpretations which were minimized by using 16 primers allowing the amplification of a sufficient number of DNA fragments for statistical analysis (Nei, 1978). The construction of a genetic distance tree based on similarity coefficients illustrates the clustering of the *C. ruminantium* isolates (Fig. 3). Whether the degree of genetic diversity observed in the genus *Cowdria* is correlated to its antigenic variability and especially cross-immunity, remains to be investigated. However, although limited, the crossprotection data reported between the isolates used in the present study are concordant with our genetic tree. The Senegal and Welgevonden stocks, which are the most distant in the genetic tree, have been shown to have very limited cross-immunity by Jongejan et al. (Jongejan et al., 1991; Jongejan et al., 1993). The same authors (Jongejan et al., 1993) also reported that 4/5, 3/5 and 2/5 goats immunized with the Senegal stock were protected against a challenge with Umpala, Lutale and Gardel stocks, respectively. These stocks are intermediate in our dendrogram between Senegal and the Welgevonden-Cameroun cluster. The antigenic relatedness of the Cameroun and the Welgevonden stocks expected from the genetic study has been confirmed by the full crossprotection obtained on 4 goats immunised by infection and treatment. The Cameroun stock also proved to be pathogenic to mice as is the Welgevonden stock.

The analysis by RAPD was completed by a Southern blot analysis to test for homogeneity of amplified fragments and to look for further polymorphisms in order to develop molecular tools that could be used directly on ticks or infected animals. The 1.2 kb fragment which was shared by the 6 stocks and therefore appeared to be a good candidate for making a genus-specific probe proved to be a mixture of at least 2 fragments, one which hybridized with a 3kb *Rsa*I fragment (Fig. 4A), and one which revealed a restriction fragment length polymorphism. This demonstrates the possibility of misinterpretation by making the assumption that RAPD fragments of the same size are homologous. In contrast, the 0.56 kb and 1.9 kb probes which were supposed to be specific for the Senegal stock, hybridized with the other stocks with different fragments of the genome. They were therefore not good candidates for stock-specific probes. A combination of the RFLP profiles obtained on Southern blots with the 1.2 kb and the 1.9 kb probes was sufficient to differentiate *C. ruminantium* isolates except Welgevonden and Cameroun which appeared identical.

In conclusion, RAPD is a suitable method for typing *C. ruminantium* isolates. It is also of value to determine the extent of the genetic diversity of this microorganism. In addition, amplified fragments can be selected to develop probes. We identified a 1.2 kb fragment that is a good genus-specific *Cowdria* probe, but were unsuccessful in finding a stock-specific probe. To circumvent this problem,

sequencing of polymorphic RAPD fragments is being conducted to try to develop genus and stock-specific PCR primers for diagnostic purposes.

Acknowledgements

This work was supported by a grant from the European Community, Directorate General XII, under contract N° TSD-CT91-0007. We thank F. Jongejan for providing the Umpala, Lutale and Senegal isolates, J.L. Du Plessis for providing the Welgevonden isolate and F. Stachurski for providing the Cameroun isolate of *Cowdria ruminantium*. We are very grateful to Professors G. Uilenberg and B.A.M. Van der Zeijst and Dr F. Jongejan for critical comments.

References

1. Provost, A. and Bezuidenhout, J.D. (1987). The historical background and global importance of heartwater. *Onderstepoort J. Vet. Res.* 54, 65-169.

2. Jongejan, F. and Uilenberg, G. (1994). Ticks and control methods. *Rev. sci. tech. Off. int. Epiz.* 13, 1201-1226.

3. Bezuidenhout, J.D. (1989). *Cowdria* vaccines, In: Veterinary protozoan and hemoparasite vaccines (Wright, I.G. Ed.), pp. 31-42. Boca Raton, CRC Press, Inc.

4. Martinez, D., Maillard, J.C., Coisne, S., Sheikboudou, C. and Bensaid, A. (1994). Protection of goats against heartwater acquired by immunisation with inactivated elementary bodies of *Cowdria ruminantium*. *Vet. Immunol. Immunopathol.* 41, 153-163.

5. Mahan, S.M., Andrew, H.R., Tebele, N., Burridge, M.J. and Barbet, A.F. (1995). Immunisation of sheep against heartwater with inactivated *Cowdria ruminantium*. *Res. Vet. Sci.* 58, 46-49.

6. Jongejan, F., Thielemans, M.J.C., Brière, C. and Uilenberg, G. (1991). Antigenic diversity of *Cowdria ruminantium* isolates determined by cross-immunity. *Res. Vet. Sci.* 51, 24-28.

7. Martinez, D., Swinkels, J., Camus, E. and Jongejan, F. (1990). Comparaison de trois antigènes pour le diagnostic de la cowdriose par immunofluorescence indirecte. *Rev. Elev. Méd. Vét. Pays Trop.* 43,159-166.

8. Van Vliet, A.H.M., Jongejan, F., Van Kleef, M. and Van der Zeijst, B.A.M. (1994). Molecular cloning, sequence analysis and expression of the gene encoding the immunodominant 32-Kilodalton protein of *Cowdria ruminantium*. *Infect. Immun.* 62, 1451-1456.

9. Mahan, S.M., McGuire, T.C., Semu, S.M., Bowie, M.V., Jongejan, F., Rurangirwa, F.R. and Barbet, A.F. (1994). Molecular cloning of a gene encoding the immunogenic 21 kDa protein of *Cowdria ruminantium*. *Microbiology*. 140, 2135-2142.

10. Lally, N.C., Nicoll, S., Paxton, E.A., Cary, C.C. and Sumption, K. (1995). The *Cowdria ruminantium* groE operon. *Microbiology*. 141, 2091-2100.

11. Reddy, G.R., Sulsona, C.R., Harrison, R.H., Mahan, S.M., Burridge, M.J. and Barbet, A.F.(1996). Sequence heterogeneity of the major antigenic protein1 genes from *Cowdria ruminantium* isolates from different geographical areas. *Clin. Diagn. Lab. Immunol.* 3, 417-422

12. Welsh, J. and McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucl. Acids Res.* 18, 7213-7218

13. Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Res.* 18, 6531-6535.

14. Uilenberg, G., Camus, E., and Barré, N. (1985). Quelques observations sur une souche de *Cowdria ruminantium* isolée en Guadeloupe (Antilles françaises). *Rev. Elev. Méd. Vét. Pays Trop.* 38, 34-42.

15. Jongejan, F., Uilenberg, G., Franssen, F.J., Gueye, A. and Nieuwenhuijs J. (1988). Antigenic differencies between stocks of *Cowdria ruminantium*. *Res. Vet. Sci.* 44, 186-189.

16. Du plessis J.L. (1985). A method for determining the *Cowdria ruminantium* infection rate of *Amblyoma hebraeum*: effect in mice injected with tick homogenate. *Onderstepoort J. Vet. Res.* 52, 55-61.

17. Cox, R.L., Kuo, C.C., Grayston, J.C. and Campbell, L.A. (1988). Deoxyribonucleic acid relatedness of *Chlamydia sp.* Twar to *Chlamydia trachomatis* and *Chlamydia psittaci*. *Int. J. Syst. Bacteriol.* 38, 265-268.

18. Mahan, S.M., Waghela, S.D., McGuire, T.C., Rurangirwa, F.R., Wassink, L.A. and Barbet, A.F. (1992). A cloned DNA probe for *Cowdria ruminantium* hybridizes with eight heartwater strains and detects infected sheep. *J. Clin. Microbiol.* 30, 981-986.

19. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloning: a Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

20. Sokal, R.R. and Michener, C.D. (1958). A statistical method for evaluating systematic relationships. *Univ. Kansas Sci. Bull.* 38, 1409-1438.

21. Ralph, D., McClelland, M., Welsh, J., Baranton, G. and Perolat, P. (1993). *Leptospira* species categorized by arbitrarily primed polymerase chain reaction (PCR) and by mapped restriction polymorphisms in PCR-amplified rRNA genes. *J. Bacteriol.* 175, 973-981.

22. Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89, 583.

23. Jongejan, F., Vogel, S.W., Gueye, A. and Uilenberg, G. (1993). Vaccination against heartwater using *in vitro* attenuated *Cowdria ruminantium* organisms. *Rev. Elev. Med. Vet. Pays Trop.* 46, 223-227.

CHAPTER 10

Development of an *in vitro* cloning method for the rickettsia *Cowdria ruminantium*

J.M. Perez [a], D. Martinez [a], A. Debus [a], C. Sheikboudou [a], A. Bensaid [b]

[a] *Centre International en Recherche Agronomique pour le Développement, Département d'Elevage et de Médecine Vétérinaire des Pays Tropicaux (CIRAD-EMVT), BP 1232, 97184 Pointe à Pitre Cedex, Guadeloupe, West Indies.*

[b] *CIRAD-EMVT, BP 5035, 34032 Montpellier, France.*

Submitted

Abstract

Cowdria ruminantium is a tick-borne rickettsia which causes severe disease in ruminants. All studies with *C. ruminantium* so far were carried out with stocks isolated directly from infected animals or the same stocks propagated *in vitro*. Cloned isolates are needed to conduct studies on immune response of the host, on genetic diversity of the parasite, and on mechanisms of attenuation and the development of vaccines. A method for the *in vitro* cloning of the organism was developed. Two hundred and sixteen clones were obtained by limiting dilution of infected cells. The method was validated by comparing RAPD fingerprints from individual clones obtained from endothelial cell cultures coinfected with 2 different stocks of *C. ruminantium*.

Cowdria ruminantium is the causative agent of cowdriosis (heartwater), an infectious disease of domestic and wild ruminants transmitted by *Amblyomma* ticks (Uilenberg, 1983). The disease is present in most African countries south of the Sahara and in several Caribbean islands from where it threatens the American mainland due to the presence of *Amblyomma* ticks which could act as vectors (Provost and Bezuidenhout, 1987; Walker and Olwage, 1987). Major economic losses are induced by fatal disease, especially in exotic breeds imported in endemic areas for genetic improvement (Uilenberg, 1995). Protection of animals can be achieved by injection of infected blood or tick homogenates followed by treatment of the reacting animals with tetracyclin (Bezuidenhout, 1989). Such vaccinated animals exhibit a prolonged and solid immunity to the homologous isolate of *C. ruminantium*. The attenuation of the Senegal isolate was obtained by serial passages in culture of endothelial cells (Jongejan, 1991), and this stock was shown to confer a good protection against the virulent homologous stock (Jongejan et al., 1993). Recently, the possibility to immunise susceptible ruminants with inactivated bacteria formulated in oil adjuvants has been described (Mahan et al., 1995; Martinez et al., 1994; 1996). However, the immunity induced by one stock of *C. ruminantium* does not necessarily protects against another stock. A great deal of research effort is now directed towards the understanding of the immune mechanisms and the development of recombinant vaccines. These vaccines should cross-protect between stocks, but there is nothing about clone diversity within and between stocks. To answer this question, methods are needed to clone *C. Ruminantium*. We have developed such a method.

The method is based on the particular life-cycle of *C. ruminantium*. *C ruminantium* is an obligate intracellular bacterium which has a Chlamydia-like developmental cycle (Jongejan et al., 1991). In the deduced replication cycle, the elementary body (EB) which represents the extracellular stage and the infectious form of the parasite, adheres to and is engulfed by endothelial cells. It remains within intracytoplasmic vacuoles where it divides by binary fission to produce a large colony named morula. After 5 to 6 days, the disruption of the host cell leads to the release of numerous EB thus initiating a new infectious cycle. In contrast to phagosomes of *Chlamydia trachomatis* which can fuse in the infected cell, the common observation of several morulae in heavily infected cell cultures suggest that *Cowdria* may have a mechanism that prevents the fusion of inclusions as occurs in *Chlamydia psittaci* (Jongejan et al., 1991). Therefore, we hypothezised that one EB develops into one morula. Thus, instead of cloning directly extracellular EB which are very labile and tend to stick strongly to cell debris, it appeared more practical to clone infected endothelial cells containing one morula.

The Gardel (Uilenberg et al., 1985) and the Senegal (Jongejan et al., 1988) stocks of *C. ruminantium* were cultivated in caprine endothelial cells (CJE cells) using previously described methods (Martinez et al., 1990). When the cell monolayers showed a cytopathogenic effect (CPE) consisting of 50-100% cell lysis, the supernatants were collected and centrifuged at 500g for 10 min to pellet cell

debris. The supernatants obtained, containing free EB, were serially diluted 2 fold in culture medium and 1 ml of each dilution was inoculated in each of 4 wells of a 24 wells microplate containing coverslips (thermanox, Nunc) coated with monolayers of CJE cells. The principle of the method was to infect endothelial cell monolayers at a low multiplicity of infection to avoid clumps of infected cells and cells containing several morulae. Since a previous study (Martinez et al., 1993) has shown that the time to the first detection of CPE was 4 to 7 days with a mean of 4.6 days, coverslips were removed and stained each day with eosin-methylen blue (RAL 555 kit), starting from day 3 after infection, to look for the presence of morulae within the cells by light microscopy. When the morulae were sufficiently developed to be easily detected (between day 3 and 5), the coverslips were thoroughly examined to determine the percentage of infected cells, to verify the presence of only one morula per infected cell and the absence of disrupted cells. The optimal dilution (1 to 10% of the cells infected by *C. ruminatium*) was thus determined and the corresponding cells used for cloning by a limiting dilution procedure. The cells were trypsinized and counted to calculate the number of infected cells per ml. These cells were then seeded at a dilution of 0.3 infected cell per well (200 µl final volume/well) in 96 wells microplates containing CJE cells. Under these conditions the first signs of cell lysis are expected after about one week in 37% of the wells according to the Poisson distribution (Debré and Seman, 1989).

As a validation of the method, endothelial cell cultures were coinfected with the Gardel and the Senegal stocks and cloned as described above. RAPD fingerprints of *C. ruminantium* (Perez et al., submitted for publication) harvested from individual positive wells were compared to typical patterns of Gardel and Senegal stocks, in order to determine whether mixed infection occured in some wells. The *Cowdria* DNA used in the PCR reaction was obtained as follows. *C. ruminantium* collected from positive wells were inoculated on CJE cells in 25 cm^2 flasks and EB were purified by differential centrifugations when 80% of the cell monolayer was lysed (Martinez et al., 1994). The suspension of EB obtained was treated with 125 µg of DNAse in buffer containing 350 µl PBS, 25 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$, in order to remove contaminating host cell DNA, as described by Cox et al. (1988). After 90 min of incubation at 37°C, the reaction was stopped by adding 25 mM EDTA and the *C. ruminantium* EB were pelleted again by centrifugation at 20,000g for 15 min at 4°C. The resultant pellet was washed three times by centrifugation in water and lysed by an overnight incubation at 55°C in a solution of 100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 25 mM EDTA, 1.5% SDS, and 250 µg/ml of proteinase K. Bacterial DNA was then extracted with phenol-chloroforme, precipitated with cold ethanol and redissolved in water. The R35 decamer primer which gives a specific pattern for each isolate of *C. Ruminantium* (Perez et al., submitted for publication) was used in the RAPD assay. The amplification reaction was performed in a volume of 25 µl containing 1.25 µl 20X polymerase buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP (New England Biolabs), 0.5 U *Tfl* DNA polymerase (Epicentre Technology), 0.2 µM of primer and

Table 1
Results of 14 cloning assays of *Cowdria ruminantium* infected cells

Assay N°	Stock of *Cowdria* cloned[a]	appearence of *Cowdria* morulae in cloned cells (in days)	% infected cells in the inoculum	% positive wells obtained
1	Gardel	4	2.0	24
2	Gardel	4	2.0	25
3	Gardel	4	0.1	5
4	Gardel	4	0.1	29
5	Gardel	3	4.0	17
6	Gardel	3	6.0	12
7	Gardel	4	4.0	2
8	Gardel	3	1.5	12
9	Senegal	5	3.0	30
10	Gardel + Senegal[b]	4	4.0	35
11	Gardel + Senegal	4	1.5	7
12	Gardel + Senegal	4	3.5	3
13	Gardel + Senegal	4	0.2	6
14	Gardel + Senegal	4	2.5	30

[a]The Gardel stock was cloned at passage 11 and the Senegal stock at passage 10 in endothelial cell culture
[b] The endothelial cells used for cloning were coinfected with the Gardel and the Senegal stocks of *C. ruminantium*

25 ng of template DNA. Following an initial denaturation of 6 min at 94°C, the PCR was carried out for 45 cycles in a Perkin Elmer Thermal Cycler (Norwalk, Conn) programmed for 1 min at 94°C, 1 min at 36°C and 2 min at 72°C, followed by a final chain extension of 10 min at 72°C. Amplified products were separated by electrophoresis in a 1.5 % agarose gel(LE Seakem, FMC Bioproducts) containing 0.5 µl/ml of ethidium bromide. Gels were photographed under UV light. Lambda Hind III digested fragments (Promega) were used as size markers.

Fourteen cloning assays were performed on cultures infected with either one stock (Gardel or Senegal) or a mixture of the Gardel and the Senegal stocks. In 58% of the positive wells, the first plaques with lysed cells were observed between 7 and 10 days after infection (Fig. 1). This corresponds to the time necessary for the EB released by a cloned infected cell introduced in a well to complete a first life-cycle whose duration is around one week (Jongejan et al., 1991; Martinez et al., 1993). No disruption of the initial infected cells can be observed microscopically. But apparently the EB released by such a cell infect neighbourous cells, resulting in a

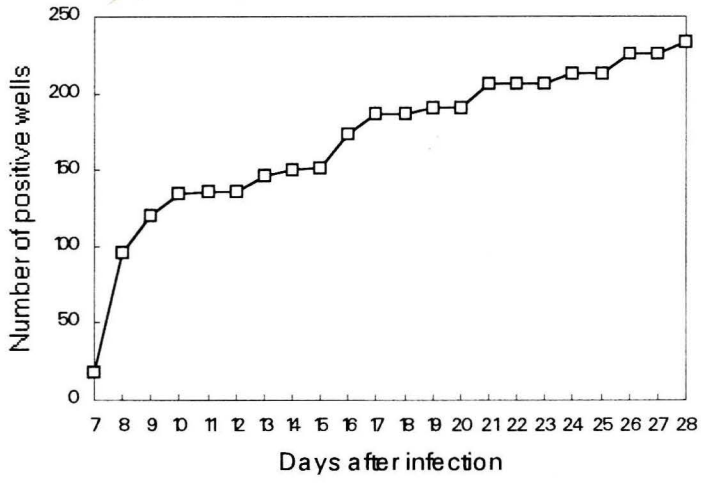


Fig. 1
Evolution of the number of positive wells (on a total of 1344 wells) obtained after cloning of *Cowdria ruminantium* in 96 well microplates coated with endothelial cells.

plaque of cell lysis. The percentage of positive wells increased slowly between day 10 and 28. These differences observed in the appearence of the first CPE suggest the existence of a great variability in the growth rate of *C. ruminantium* clones. The maximum frequency of 35% of positive wells obtained in the assays (Table 1) corresponds to a probability of 0.03 that two *C.ruminantium* EB were distributed in the same well, according to the Poisson distribution. In most assays, this probability was even lower since the frequency of positive wells was less than 30%. This predicts that most positive wells should contain a clonal population of *C. ruminantium*. The fact that in most experiments, the percentage of positive wells was lower than the expected value of 35% can be due to an infectivity of EB lower than 100% and inaccuracy in the measurement of the fraction of infected cells used for cloning. In 17 out of 233 positive wells 2 plaques of lysis appeared simultaneously. These wells were probably infected by 2 clones. In addition, in assays 10 and 14 (Table 1), cultures coinfected with the Gardel and Senegal isolates were cloned and the resultant clones characterized by RAPD. No case of mixed infection was detected in the same well. In assay 10, 18 clones displayed a Senegal fingerprint (data not shown), and in assay 14, amongst 25 clones analysed, 13 displayed a typical Senegal pattern and 12 a typical Gardel pattern (Fig. 2).

This is the first report of a method for cloning the intracellular pathogen *C. ruminantium*. The result show that the method developed in this study consisting of cloning infected cells instead of extracellular parasites, is of value to obtain clones of *C. ruminantium*. Cloning extracellular *C. ruminantium* by a limiting dilution or a

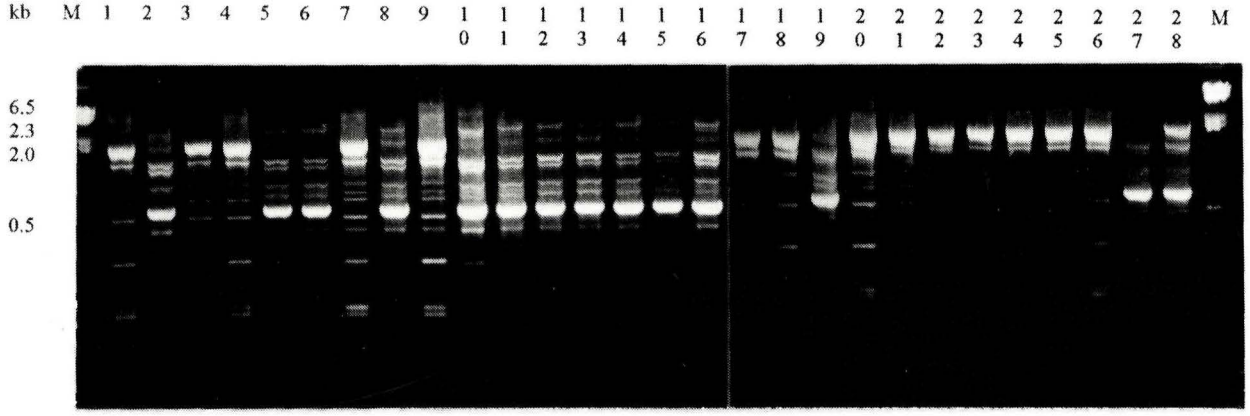


Fig. 2
RAPD fingerprints of *Cowdria ruminantium* clones obtained with primer R35. Lanes 26, 27 and 28 represent RAPD pattern of Senegal, Gardel and a mixture of Senegal and Gardel stocks, respectively. Lanes 1 to 25 represent RAPD patterns of individual clones obtained after cloning of a culture coinfected with Senegal and Gardel stocks. Lanes M are molecular size markers in kilobases.

plaque method would have probably been possible. However, a great advantage of our method is that each step of the procedure can easily be monitored. Indeed, the evaluation of the number of infected cells and therefore the determination of the optimal dilution for cloning was easy, compared to the great difficulty to quantitate the number of living EB in a suspension. The verification of the absence of multiple infection in a single cell was possible by a simple microscopic examination. Finally, a routine examination of microplates under the inverted microscope allowed the detection and the subsequent elimination of the wells showing the simultaneous development of more than 1 plaque of cell lysis which probably were infected by more than one clone. Clones can be obtained in one single assay whereas with usual limiting dilution procedures it is advised to repeat 2 or 3 times the cloning assay (Debré and Seman, 1989). As in previous studies conducted on *Babesia* (Carson et al., 1990; Gill et al., 1987; Timms et al., 1990) and *Plasmodium* models (Fenton et al., 1985; Knowles and Walliker, 1980), *C. ruminantium* clones can now be compared in terms of vector transmission, pathogenicity, DNA polymorphism, protein and antigen composition. These studies will be relevant for the study of immune mechanisms by using characterised clones instead of whole isolates, and the evaluation of the extent of clone diversity within and between isolates. By cloning the Senegal stock it would also be possible to verify whether its attenuation is the result of a selection of clones with avirulent phenotype during passaging as demonstrated in *Babesia bovis* (Dalrymple, 1992), or if it is due to another genetic mechanism.

Aknowledgements

This work was supported by a grant from the European Community, Directorate General XII, under contract N° TSD-CT91-0007. We are very grateful to Professors G. Uilenberg, B. Van der Zeijst and to Dr F. Jongejan for useful comments on the manuscript.

References

1. Bezuidenhout, J.D. 1989. *Cowdria* vaccines, p. 31-42. In I.G. Wright, Veterinary protozoan and hemoparasite vaccines. Boca Raton, CRC Press, Inc.

2. Carson, C.A., P. Timms, A.F. Cowman, and N.P. Stewart. 1990. *Babesia bovis*, evidence for selection of subpopulations during attenuation. *Exp. Parasitol.* 70, 404-410.

3. Cox, R.L., C.C. Kuo, J.T. Grayston, and L.A. Campbell. 1988. Deoxyribonucleic acid relatedness of *Chlamydia sp.* Twar to *Chlamydia trachomatis* and *Chlamydia psittaci*. *Int. J. Syst. Bacteriol.* 38, 265-268.

4. Dalrymple, B.P. 1992. Diversity and selection in *Babesia bovis* and their impact on vaccine use. *Parasitol. Today* 8, 21-23.

5. Debré, P., and M. Seman. 1989. Clonage, p. 35-41. *In* INSERM (ed.), Clonage des lymphocytes T. Société française d'Immunologie.

6. Fenton, B., A. Walker, and D. Walliker. 1985. Protein variation in clones of *Plasmodium falciparum* detected by two dimensional electrophoresis. *Mol. Biochem. Parasitol.* 16, 173-183.

7. Gill, A.C., A.F. Cowman, N.P. Stewart, D.J. Kemp, and P. Timms. 1987. *Babesia bovis*, molecular and biological characteristics of cloned parasite lines. *Exp. Parasitol.* 63, 180-188.

8. Jongejan, F. 1991. Protective immunity to heartwater (*Cowdria ruminantium* infection) is acquired after vaccination with *in vitro* attenuated *Rickettsiae*. *Infec. Immun.* 59, 729-731.

9. Jongejan, F., G. Uilenberg, F.J. Franssen, A. Gueye, and J. Nieuwenhuijs. 1988. Antigenic differences between stocks of *Cowdria ruminantium*. *Res. Vet. Sci.* 44, 186-189.

10. Jongejan, F., S.W. Vogel, A. Gueye, and G. Uilenberg. 1993. Vaccination against heartwater using *in vitro* attenuated *Cowdria ruminantium* organisms. *Rev. Elev. Méd. Vét. Pays Trop.* 46, 223-227.

11. Jongejan, F., T.A. Zandbergen, P.A. Van De Wiel, M. De Groot, and G. Uilenberg. 1991. The tick-borne Rickettsia *Cowdria ruminantium* has a *Chlamydia*-like developmental cycle. *Onderstepoort J. vet. Res.* 58, 227-237.

12. Knowles, G., and D. Walliker. 1980. Variable expression of virulence in the rodent malaria parasite *Plasmodium yoelii yoelii*. *Parasitology* 81, 211-219.

13. Mahan, S.M., H.R. Andrew, and N. Tebele. 1995. Immunisation of sheep against heartwater with inactivated *Cowdria ruminantium*. *Res. Vet. Sci.* 58, 46-49.

14. Martinez, D., J.C. Maillard, S. Coisne, C. Sheikboudou, and A. Bensaid. 1994. Protection of goats against heartwater acquired by immunisation with inactivated elementary bodies of *Cowdria ruminantium*. *Vet. Immunol. Immunopathol.* 41, 153-163.

15. Martinez, D., J.M. Perez, C. Sheikboudou, A. Debus, and A. Bensaid. 1996. Comparative efficacy of Freund's and Montanide ISA 50 adjuvants for the immunisation of goats against heartwater with inactivated *Cowdria ruminantium*. *Vet. Parasitol.*, 67, 175-184.

16. Martinez, D., C. Sheikboudou, P.O. Couraud, and A. Bensaid. 1993. *In vitro* infection of bovine brain endothelial cells by *Cowdria ruminantium*. *Res. Vet. Sci.* 55, 258-260.

17. Martinez, D., J. Swinkels, E. Camus, and F. Jongejan. 1990. Comparaison de trois antigènes pour le diagnostic de la cowdriose par immunofluorescence indirecte. *Rev. Elev. Méd. Vét. Pays Trop.* 43, 159-166.

18. Perez, J. M., D. Martinez, A. Debus, C. Sheikboudou, and A. Bensaid. Detection of genomic polymorphisms among isolates of the intracellular bacterium *Cowdria ruminantium* by random amplified polymorphic DNA and Southern blotting (submitted)

19. Provost, A., and J.D. Bezuidenhout. 1987. The historical background and global importance of heartwater. *Onderstepoort J. vet. Res.* 54, 165-169.

20. Timms, P., N.P. Stewart, and A.J. De Vos. 1990. Study of virulence and vector transmission of *Babesia bovis* by use of clone parasite lines. *Infect. Immun.* 58, 2171-2176.

21. Uilenberg, G. 1983. Heartwater (*Cowdria ruminantium* infection), Current status. *Adv. vet. Sci. comp. Med.* 27, 427-480.

22. Uilenberg, G. 1995. International collaborative research, significance of tick-borne hemoparasitic diseases to world animal health. *Vet. Parasitol.* 57, 19-41.

23. Uilenberg, G., E. Camus, and N. Barré. 1985. Quelques observations sur une souche de *Cowdria ruminantium* isolée en Guadeloupe (Antilles françaises). *Rev. Elev. Méd. Vét. Pays Trop.* 38, 34-42.

24. Walker, J.D., and A. Olwage. 1987. The tick vectors of *Cowdria ruminantium* (Ixodoidea, Ixodidae, genus *Amblyomma*) and their distribution. *Onderstepoort J. vet. Res.* 54, 353-379.

CHAPTER 11

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

In this thesis research towards two main goals is described.

- We have successfully immunised ruminants with an inactivated preparation of *C. ruminantium* and we have contributed to the understanding of the protective immune mechanisms thus opening the way for research on a recombinant vaccine.

- We have undertaken an evaluation of the extent of genetic diversity of *Cowdria* both between and within isolates by developing an RAPD assay and a method for cloning the parasite.

Development of inactivated vaccines

In two successive experiments, two subcutaneous injections of inactivated elementary bodies of *C. ruminantium* formulated in Freund's adjuvant (FA) were shown to protect a total of 6 out of 9 susceptible goats against a heavy homologous challenge administered intravenously 3.5 months after vaccination (chapter 3). The effectiveness of immunizing with killed *Cowdria* formulated in Freund's adjuvant was thereafter confirmed in cattle (chapter 7). Five out of 5 vaccinated animals were solidly immune against an homologous challenge. From these 5 cattle, 3 survived a lethal challenge 10 months after immunisation. The better level of protection obtained in cattle than in goats, was probably due to the lower susceptibility of this species, as already reported from immunisations with infection and treatment.

Complete and incomplete Freund's adjuvants used in these trials are very potent experimental immunoenhancers which unfortunately have important side-effects making them unsuitable for the preparation of a commercial vaccine even in veterinary medicine (Edelman, 1980). Amongst the variety of licenced adjuvants, Montanide ISA 50 has proved to be effective in immunisation against various microorganisms. This adjuvant based on mannide monooleate emulsifying agent in purified mineral oil, was chosen as the most likely to give an immune response similar to that induced by Freund's adjuvant. In an additional experiment conducted on goats, the levels of protection confered by water-in-oil emulsions of inactivated *Cowdria* in these two adjuvants were compared (chapter 6). Both adjuvants proved equivalent, protecting 3 out of 5 goats against a lethal challenge administered 3.5 months after vaccination. In addition, in the case of the vaccine formulation with Montanide ISA 50, the establishment of a long lasting immunological memory was demonstrated by the survival of 3 out of 6 and 5 out of 6 immunised goats that survived the challenge 15 and 17 months after the last vaccination respectively.

During the vaccination experiments on goats, it was observed that the incubation period and the development of the disease were affected by the infective dose of the challenge. Highly infective doses consisting of 2 to 5 ml of infectious supernatant shortened the incubation period to an average of 6 $\pm$ 1.5 days (N=37) compared with average incubation period of 10 to 14 days usually obtained with a tick challenge or the injection of infective blood. By decreasing the dose to 50 and 10 TCLD50 per goat for the challenges at 15 and 17 months respectively, an incubation of 11 days was obtained in the controls, and this led to an increase in the survival rate of vaccinated goats. Although the average tick-delivered challenge in the field is unknown, we can postulate that such a challenge was approached by decreasing the dose until a naturally occuring incubation period was reached, and that this dose is most useful to test experimental vaccines.
Standardization of the virulent inocula appears essential to compare the efficacy of vaccinal preparations within and between laboratories, and also to compare the virulence of different stocks and conduct cross-immunization studies.

These results constitute the first report of a successful immunisation of ruminants against *Cowdria* using killed antigens. If all the assays are combined irrespective of the adjuvant used, the dose, or the timing of the challenge, 100% (N=5) of the cattle were protected, 65% (N=31) of the vaccinated goats survived, whereas 100% (N=30) of the naive or adjuvant controls died. This method of vaccination which has also protected sheep (Mahan et al., 1995) is a significant improvement on the infection and treatment method used to date. The long lasting and the good levels of protection obtained with a dose similar to that experienced by the animals under natural conditions suggest that a field challenge may efficiently boost the immune response of vaccinated animals. Since the method is based on killed antigens, it can be used in areas where the disease is not endemic, and any stock of *Cowdria* can potentially be incorporated in the vaccine to cover the antigenic diversity in the field. Finally, since Montanide ISA 50 is a licenced adjuvant, a water in oil emulsion of killed *Cowdria* antigens in this adjuvant can be considered has a first generation inactivated vaccine against cowdriosis ready to be tested in the field.

Improvements are, however, needed. It is likely that the dose of inactivated *Cowdria* can be reduced, but a minimum dose remains to be determined. Even though the dose of antigen can be decreased, a mass production of endothelial cells will still be needed to produce it. This could be achieved by developing mass culture of these cells and *Cowdria* in bioreactors.
Montanide ISA 50 can also induce local adverse inflammatory reactions in goats. Besides the determination of the minimum dose of *Cowdria* antigen, it is therefore important to determine the optimum volume of adjuvant and the best route of inoculation. Results in cattle and preliminary studies in goats show that intramuscular injections give lower local reactions than subcutaneous injections.

Alternatively, Montanide adjuvants containing a metabolisable oil instead of mineral oil have been developed to overcome adverse reactions, and could be tested.

On the long term, research will be focused on the identification of protective antigens which can be produced by DNA recombinant technology. If peptide vaccines are to be used, a better adjuvant is essential to overcome the generally weak immunogenicity of such vaccines (Audibert and Lise, 1993). Another approach consists with delivery of antigens through the expression by replicating viral or bacterial vectors which are very immunogenic and have the ability to efficiently trigger both $CD4^+$ and $CD8^+$ T cell responses. Amongst the microbial delivery systems available, the capripoxvirus which has already shown to be able to protect cattle against rinderpest (Romero et al., 1993), would be of great interest for cowdriosis. In effect, the virus is endemic in African regions where heartwater occurs, has a limited host range, is non pathogenic for humans, is heat stable, and will offer protection against lumpy skin disease, caprine poxvirus and possibly other diseases like rinderpest if genes for protective antigens are included. DNA immunization which is being studied in a number of microbial models, may also be a promising approach for vaccine design (Braciale, 1993). Vaccination with such naked DNA has been shown to induce both humoral and cell-mediated immune responses which often provide protective immunity (Hasset and Whitton, 1996).

Analysis of the immune response to *Cowdria*

Successful vaccination depends on the induction of an appropriate immune response capable of protecting the host against the disease when the causative organism is encountered later. Immune response against a pathogen can lead to protection or pathology depending on a complex regulation. It is now clear that Th1 and Th2 lymphocyte sub-populations play a pivotal role in this regulation for many microbial and parasitic infections (Mosmann and Sad, 1996). Thus, although this is not an absolute rule, generally Th1 responses are protective against intracellular parasites, whereas Th2 responses are associated with progressive disease. Conversely, protection against helminths is generally associated with a Th2 type response (Cox and Liew, 1992). A study of the immune response in cowdriosis was undertaken in order to identify the protective mechanisms and therefore to be able to use proper tests for the screening of relevant antigens for vaccination.

Since immunity to *Cowdria* is not correlated to the level of circulating antibodies, and there is no protection *in vivo* by the transfer of immune serum, it is generally assumed that antibodies play little role in protection against *Cowdria* infection. However, there are two reports by Du Plessis (1993) and Byrom et al. (1993) that a degree of neutralization with antiserum can be achieved *in vitro*. In our study, immune sera from goats immunized by infection and treatment or by inactivated

Cowdria in adjuvant, were unable to neutralize the infection of bovine endothelial cells by *Cowdria in vitro* (chapter 3). However, a certain role of antibodies cannot be completely excluded. In particular, the possible influence of the isotype of immunoglobulins, and the involvement of specific antibodies in antibody dependent cytotoxicity reactions need to be investigated.

A first approach for the understanding of immune mechanisms was performed on an *in vitro* model of *Cowdria* infection. The tropism of *Cowdria* for brain capillaries was verified by infecting bovine brain endothelial cells (BBEC) *in vitro* (chapter 2). These cells did not show significant differences in their ability to support *Cowdria* growth when compared to bovine umbilical endothelial cells (BUE), and were used to study the synthesis of proinflammatory cytokines and MHC transcripts upon infection by *Cowdria* in the presence or absence of IFN-γ (chapter 4). The constitutive levels of MHC class I BoLA A and B mRNA was elevated after treatment with IFN-γ but was not affected by *Cowdria* infection. The levels of MHC class II DQβ, DRα, DRβ and Ii mRNA induced by IFN-γ did not change after *Cowdria* infection. However, *Cowdria* infection selectively elicited the expression of a DQα transcript of an unusual 1.5 kb size clearly distinct from the 1.3 kb IFN-γ-induced transcript. Although the role of this transcript was not elucidated, this suggests an up-regulation of DQα mRNA by *Cowdria* infection.
From these data it was tempting to speculate that MHC class II expression is induced on BBEC during *Cowdria* infection which has in turn, important effects on the generation of specific $CD4^+$ T cells. However, flow cytometry analysis showed an inhibition of the expression of MHC class II molecules at the surface of BUE and caprine jugular endothelial cells (CJE) treated with IFN-γ, upon infection by *Cowdria* (chapter 5). This suggests that *Cowdria*, like many other intracellular pathogens (Smith, 1994), has developed a mechanism to evade the immune response of the host by blocking the transit of MHC class II molecules at the cell surface. An extension of this study with BBEC cells focussed on the expression of MHC class I molecules that have a role in the presentation of microbial peptides to $CD8^+$ T cells is underway.

The infection of BBEC by *Cowdria* in synergy with IFN-γ induced the synthesis of IL-1β, IL-6 and IL-8 mRNA (chapter 4). IL-1 and IL-6 are known to be involved in the induction of fever which is a constant symptom in heartwater. IL-1 receptors are widespread in the brain, particularly in post-capillary veinules (Cunningham and De Souza, 1993), suggesting that IL-1 is be implicated in the increased permeability of brain capillaries and the up-regulation of adhesion molecules expression, thus favoring leucocyte infiltration. The synthesis of IL-8 might participate in the recruitment of neutrophils with potentially deleterious effects. On the other hand, IL-1 and IL-6 may also sustain the activation of *C. ruminantium* specific T cells and the production of antibodies. It is likely that these cytokines are also responsible at

least in part, for the increased vascular permeability in other organs affected in cowdriosis, such has lungs and pericardium, but this remains to be demonstrated.

These studies conducted *in vitro* indicate that BBEC may be able to present antigens to T cells and thus contribute to the development of an immune response against *Cowdria* which in turn has developed strategies to inhibit this presentation. Immunohistochemical and *in situ* hybridization studies for cytokines and MHC molecules conducted *ex vivo* on organs of infected ruminants are necessary to confirm such mechanisms.

An analysis of the T cell response *in vivo* was performed in cattle and goats vaccinated with killed *Cowdria* in oil adjuvant. The characterization of the proliferative response of lymphocytes to *Cowdria* antigen was performed in cattle (chapter 7). Peripheral blood mononuclear cells (PBMC) from all immunized animals responded strongly *in vitro* to *Cowdria* antigens before the challenge. Incubation of PBMC with *Cowdria* antigen resulted in an increase in relative cell number in the $CD4^+$ fraction only. In addition, only this fraction showed an increase of both MHC class II and IL-2 receptor which is consistent with an active proliferation. Other cell types also expressed higher MHC class II molecules but did not up-regulate IL-2 receptor expression. When PBMC were incubated with lysates of endothelial cells which might have contaminated the vaccinal preparations, the majority of responding cells were γδ lymphocytes. Analysis by flow cytometry of short-term T cell lines generated *in vitro* by three restimulations at weekly intervals, showed that they were 95 to 100% $CD4^+$ T lymphocytes. Blocking experiments with monoclonal antibodies confirmed that proliferation was MHC class II restricted, and the absence of proliferation in presence of heterologous antigen presenting cells proved that the proliferation was genetically restricted. In addition, upon stimulation with *Cowdria* antigen, T cell lines secreted significant levels of IFN-γ which was shown to be a potent inhibitor of *Cowdria* growth *in vitro* (chapter 5). Screening of protein fractions of *Cowdria* with these specific $CD4^+$ T cell lines is being done to identify candidate antigens for a vaccine.

The evolution of the phenotype of PBMC subpopulations obtained from goats and cattle was carried out by flow cytometry (chapter 8). Data were expressed either in percentage of total PBMC after the first challenge (CH1) of goats vaccinated as described in chapter 6 or in absolute cell count for cattle or goats challenged with 50 (CH2) or 10 TCLD50 (CH3) of *Cowdria*. No significant variations were shown during the process of immunisation. After all challenges except CH3, a gradual decline in total leucocyte count was observed during 10 days, after which numbers increased to preinfection levels by day 13 to 14. Blood monocytes increased greatly soon after challenge for cattle and goats vaccinated with a vaccine containing Freund's adjuvant and remained at high levels until day 16-17. The increase occured

later, between day 10 and 17, for animals that received an ISA 50 containing vaccine. At microscopic examination, monocytes appeared strongly activated, resembling macrophages with enlarged size, polylobulated nuclei and vacuolated cytoplasm. In cattle, the level of $CD4^+$ cells, B cells and at a lesser extent $CD8^+$ cells dropped during 10 days, recovering preinfection values after 3 to 4 weeks post inoculation. No significant change was observed in the level of γδ T cells. In goats, a similar drop was observed for $CD4^+$, $CD8^+$ and γδ T cells. A considerable increase in the number of $CD8^+$ lymphocytes was observed from day 13 to 37 approximately. At the peak occuring around day 17, the percentage of $CD8^+$ was more than 50% of total PBMC. These $CD8^+$ cells were characterized in cattle as αβ T cells. There was a 2.5- to 3- fold increase of blasts at the peak. From these observations, a significant role for $CD8^+$ T lymphocytes in the resistance to cowdriosis can be assumed. However, it should be pointed out that the number of $CD8^+$ cells increases only after 2 weeks, by which time control animals are dead. Other mechanisms of protection may therefore take place before the $CD8^+$ response is generated.

The results on the T lymphocyte response strongly suggests a Th1-type protective immune response in cowdriosis with an important role of INFγ secreted by activated $CD4^+$ T cells. The importance of INFα demonstrated both *in vitro* and *in vivo* in *Cowdria* infection (Totté et al., 1993) is in favor of this hypothesis, since this cytokine is known to play an important role in T-cell differentiation towards a Th1 type of immune response (Belardelli and Gresser, 1996). The important increase of $CD8^+$ T cells after virulent inoculation, indicates that both $CD4^+$ and $CD8^+$ T lymphocyte subsets are probably implied at various degrees in the immune response to cowdriosis, as for other intracellular bacterial or protozoal infections (Gazzinelli et al., 1991; Kaufman, 1995). In *Cowdria* infection, it remains to be shown whether the $CD4^+$ population activated by the immunization responds *in vivo* to live *Cowdria* challenge, and whether the $CD8^+$ T cells have a cytotoxic activity. These follow-up studies are underway, but an antigen dependant and genetically restricted cytotoxicity has already been shown on preliminary experiments conducted on two goats. Also, monozygotic goat and sheep twins have been produced by embryo-splitting and transfer (Chesné et al., 1995). They will serve in immune cell transfer experiments to determine the respective role of T cell populations in protection.
The role of monocytes which peak and are strongly activated soon after challenge needs to be investigated. Macrophages are known to play a crucial role in early protection against infection by intracellular bacteria (Daugelat et al., 1994) and to secrete a large variety of cytokines. They are in particular, potent producers of IL-12 which acts as an inducer of IFNγ by NK and T cells, and favor differentiation of T cells towards a Th1 phenotype (Trinchieri, 1995).
Finally, immunological studies were all conducted on blood cells which may not represent accurately the response. A study on the lymph draining a tick bite should also be conducted.

The diversity of *Cowdria*

A major problem for the development of vaccines is to overcome the antigenic diversity of the pathogens. These pathogens are engaged in a dynamic coevolutionary interaction with their host population, whose immune system represents a major selective force that shape the phenotype of the parasites (Brunham et al., 1993). Cross-protection studies have revealed the existence of an important antigenic diversity of *Cowdria*, the extent of which needs to be evaluated for the development of vaccines. Until recently, no method except cross-immunization was available to characterize this diversity.

A random amplified polymorphic DNA assay (RAPD) was developed for *Cowdria* and evaluated by typing 6 isolates originating from widely distant geographical regions with 16 primers (chapter 9). Distinction among stocks was possible by using one or at the most two primers, but two stocks always presented very similar fingerprints. The genetic diversity of *Cowdria* appeared important since only 24% out of 108 fragments generated by the 16 primers were common among stocks. A restriction fragment length polymorphism pattern obtained by probing several RAPD fragments on *RsaI* digest of genomic DNA of the 6 isolates, made it possible to differentiate the except two stocks that were similar in RAPD analysis. A genetic tree was constructed from the RAPD analysis. Although the diversity determined by genetic analysis does not necessarily correlate with the antigenic diversity involved in cross-protection, the results obtained in our study were promising. Both stocks that were genetically very closely related, were also fully cross-protective and similarly pathogenic to mice. The most distant stocks are known to give a very little cross-protection, and isolates which have an intermediate genetic distance also present an intermediate ability to protect one to the other. An analysis of more isolates is underway to look whether clustering of stocks based on a genetic analysis will be of help in predicting the number of antigenically different groups to test by cross-immunization. For this purpose, our approach consisting of analysing genetic polymorphisms widely and randomly dispersed in the *Cowdria* genome is probably more appropriate than typing strains by a PCR-RFLP method on a highly conserved gene. The disadvantages of RAPD is that it requires isolation of pure DNA which is difficult for *Cowdria*, and is difficult to standardize between laboratories. The RAPD technique can also be used to identify genus- or isolate-specific fragments to develop probes or PCR primers for diagnostic purposes. Testing of putative interesting fragments for diagnostic, as well as a systematic sequencing of RAPD fragments for research of *Cowdria* genes are underway.

So far, all studies on cowdriosis have been carried out with isolates (stocks), but it is not known how heterogeneous isolates are. A method for cloning *Cowdria in vitro*

was thus developed (chapter 10). It was based on the particular *Chlamydia*-like life cycle of *Cowdria*. An elementary body (EB), the infectious form of *Cowdria*, is engulfed by the endothelial cell within a cytoplasmic vacuole where it divides to give a colony (morula). As for *Chlamydia psittaci*, *Cowdria* seems to have a mechanism preventing the fusion of inclusions when several EB enter the host cell. Thus, it appeared easier to clone endothelial cells containing only one morula of *Cowdria* instead of cloning extracellular EB. The method was validated by identifying individual clones obtained from cell cultures coinfected with two different stocks of *Cowdria*, by their RAPD fingerprint. Clones will be useful for the study of immune responses and to understand the mechanism of attenuation. Until recently, only the Senegal stock had been successfully attenuated *in vitro*, but we have now succeeded in also attenuating the Gardel stock (Martinez and Sheikboudou, unpublished data) which will be useful for the study of the attenuation.

Studies are planned to verify whether the attenuation obtained *in vitro* is the result of a selection of avirulent clones preexisting within the isolate, or if it is due to another genetic mechanism. With our method, it is possible to obtain clones from low and high passaged (attenuated) Senegal and Gardel stocks, and compare them in terms of vector transmission, pathogenicity, DNA polymorphism, and antigen composition.

Both attenuated Senegal and Gardel stocks confer a good protection against an homologous challenge, and they have a complementary spectrum of cross-protection with other isolates. In addition, preliminary studies conducted in goats have shown that these avirulent stocks are not transmitted by *Amblyomma* ticks. Thus it may be possible to select clones unable to be transmitted by ticks and with low pathogenicity, to formulate live attenuated vaccines.

References

1. Audibert, F.M., Lise, L.D., 1993. Adjuvants : current status, clinical perspectives and future prospects. *Immunol. Today* 14, 281-284.

2. Braciale, T.J., 1993. Naked DNA and vaccine design. *Trends Microbiol.* 9, 323-325.

3. Brunham, R.C., Plummer, F.A., Stephens, R.S., 1993. Bacterial antigenic variation, host immune response, and pathogen-host coevolution. *Infect. Immun.* 61, 2273-2276.

4. Byrom, B., Mahan, S.M., Barbet, A.F., 1993. The development of antibody to *Cowdria ruminantium* in mice and its role in heartwater disease. *Rev. Elev. Méd. Vét. Pays trop.* 46, 197-201.

5. Chesné, P., Cognié, Y., Martinez, D., Depres, E., Aumont, G., 1995. Production of identical tropical goat twins for pathology research. *11th European Association for Embryo Transfer meeting*, Hannover, 8-9 september 1995, p 144.

6. Cox, F.E.G., liew, F.Y., 1992. T-cell subsets and cytokines in parasitic infections. *Immunol. Today* 13, 445-448.

7. Cunningham, E.T., De Souza, E.B., 1993. Interleukin 1 receptors in the brain and endocrine tissues. *Immunol. Today* 14, 171-176.

8. Daugelat, S., Ladel, C.H., Schoel, B., Kaufmann, S.H.E., 1994. Antigen-specific T-cell response during primary and secondary *Listeria monocytogenes* infection. *Infect. Immun.* 62, 1881-1998.

9. Du Plessis, J.L., 1993. An *in vitro* test to demonstrate the inhibitory effect of homologous immune serum on the infectivity of *Cowdria ruminantium*. *Onderstepoort J. vet. Res.* 60, 69-73.

10. Edelman, R., 1980. Vaccine adjuvants. *Rev. Infect. Dis.* 2, 370-383.

11. Gazzinelli, R.T., Hakim, F.T., Hieny, S., Shearer, G.M., Sher, A., 1991. Synergistic role of $CD4^+$ and $CD8^+$ T lymphocytes in IFNγ production and protective immunity by an attenuated *Toxoplasma gondii* vaccine. *J. Immunol.* 146, 286.

12. Hasset, d.h., whitton, j.l., 1996. DNA immunization. *Trends Microbiol.* 4, 307-312.

13. Kaufman, S.H.E., 1995. Immunity to intracellular microbial pathogens. *Immunol. Today* 16, 338-342.

14. Mosmann, T.R., sad, S., 1996. The expanding universe of T-cell subsets : Th1, Th2 and more. *Immunol. Today* 17, 138-146.

15. Romero, C.H., Barrett, T., Evans, S.A., Kitching, R.P., Gershon, P.D., Bostock C., Black, D.N., 1993. Single capripox recombinant vaccine for the protection of cattle against rinderpest and lumpy skin disease. *Vaccine* 11, 737-742.

16. Smith, G.L., 1994. Virus strategies for evasion of the host response to infection. *Trends Microbiol.* 2, 81-88.

17. Totté, P., Jongejan, F., De Gee, A.L.W., Wérenne, J., 1994. Production of alpha interferon in *Cowdria ruminantium*-infected cattle and its effect on infected endothelial cell cultures. *Infect. Immun.* 62, 2600-2604.

18. Trinchieri, G., 1995. Interleukin-12 : a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Ann. Rev. Immunol.* 13, 251-276.

RESUME

Dans ce travail de thèse, nous avons effectué des essais de vaccination sur ruminants à l'aide de diverses préparations antigéniques de *Cowdria ruminantium*, et analysé *in vitro* les réponses immunitaires protectrices induites. Par ailleurs, des méthodes ont été développées afin d'appréhender la variabilité génétique de l'agent pathogène.

Dans deux expérimentations successives, nous avons montré que deux injections de corps élémentaires inactivés de *C. ruminantium* émulsifiés dans de l'adjuvant de Freund permettaient de protéger des chèvres contre une inoculation létale pour 100% des animaux témoins. Ce procédé d'immunisation s'est également montré très efficace pour protéger des bovins. L'adjuvant de Freund étant réservé à des essais expérimentaux en raison des effets secondaires qu'il entraine, nous avons dans une troisième expérimentation comparé l'efficacité de deux préparations vaccinales de type émulsion « eau dans l'huile » dont la phase aqueuse était constituée de corps élémentaires de *Cowdria* dans du tampon phosphate, et la phase huileuse d'adjuvant de Freund ou de Montanide ISA 50. Le Montanide ISA 50, adjuvant huileux agrée pour la formulation de vaccins animaux, s'est montré aussi efficace que l'adjuvant de Freund. Les deux types de préparations vaccinales ont permis de protéger trois chèvres sur cinq contre une épreuve virulente administrée 3,5 mois après la vaccination. La durée de la protection induite chez les chèvres par le vaccin à base de Montanide ISA50 peut être considérée comme bonne. Trois chèvres sur six, et cinq chèvres sur six ont survécu à des épreuves virulentes effectuées respectivement 15 et 17 mois mois après les injections de rappel. Chez les bovins, le Montanide ISA50 n'a pas été testé, mais la préparation à base d'adjuvant de Freund a permis de protéger trois bovins sur trois, 10 mois après leur vaccination. Sur l'ensemble des essais, 64% des chèvres vaccinées (N = 31) et tous les bovins (N = 5) ont été protégés alors que tous les animaux témoins (30 chèvres et 3 bovins) sont morts.

Les expérimentations sur animaux ont révélé l'influence de la concentration en organismes virulents sur l'évolution clinique de la maladie et le taux de survie des chèvres vaccinées. Dans les deux premiers essais de vaccination sur chèvres et dans la première partie du troisième essai, les doses d'épreuves (2 à 5 ml de surnageant de culture administrés par voie intraveineuse) se sont révélées beaucoup trop élevées. Elles ont induit des périodes d'incubation de 5 jours environ alors que l'incubation normale est en moyenne d'une douzaine de jours. Dans la deuxième partie du troisième essai, la dose d'épreuve a été diminuée à 50 puis à 10 TCLD50 (dose létale 50% en culture de tissus). La dose de 10 TCLD50 a permis d'obtenir une période d'incubation de 11 jours, durée proche de celle observée dans les conditions

naturelles. Il est nécessaire dans l'avenir de parvenir à calibrer la dose d'épreuve à un niveau aussi proche que possible d'une dose naturelle pour évaluer l'efficacité des vaccins.

La vaccination à l'aide de bactéries tuées, constitue une amélioration importante par rapport à la méthode d'immunisation par infection et traitement. Les bons niveaux de protection obtenus ainsi que la durée appréciable de l'immunité induite par la préparation vaccinale à base de Montanide ISA 50, permettent de la considérer comme un vaccin de première génération pouvant être évalué à large échelle dans des conditions de terrain. Les recherches se focalisent à présent vers l'identification des antigènes protecteurs candidats pour le développement d'un vaccin recombinant.

Nous avons dans un deuxième temps effectué une étude des mécanismes immunitaires et pathologiques mis en jeu lors d'une infection par *Cowdria* en nous attachant particulièrement aux ruminants vaccinés par des microorganismes inactivés puis éprouvés par inoculation de *Cowdria* virulentes.

Les sérums d'animaux ayant survécu à une épreuve virulente après immunisation par infection-traitement ou par injection de *Cowdria* inactivées dans l'adjuvant de Freund, n'ont pas permis de neutraliser le pouvoir infectant de *Cowdria* pour des cellules endothéliales *in vitro*. Ces résultats sont en accord avec l'absence de protection conférée à des ruminants par le transfert de sérum hyperimmun et nous ont conduit à privilégier l'étude des réponses cellulaires.

Une étude a été conduite *in vitro* sur des cellules endothéliales de vaisseaux capillaires cérébraux de bovins (BBEC) qui constituent une cible privilégiée pour *Cowdria*. Cette étude a montré que l'infection des BBEC par *Cowdria* en synergie avec un traitement par l'interferon γ, induisait la synthèse des ARNm de l'IL-1, de l'IL-6 et de l'IL-8 (mise en évidence par RT-PCR). Ces cytokines pro-inflammatoires pourraient intervenir dans les phénomènes de fièvre et l'augmentation de la perméabilité des vaisseaux capillaires observés dans la cowdriose. L'IL-8 pourrait pour sa part être impliquée dans le recrutement de neutrophiles dans les lésions inflammatoires avec induction d'effets délétères. L'infection par *Cowdria* n'affecte pas le niveau de transcription des gènes BoLA A et B du Complexe Majeur d'Histocompatibilité (CMH) de classe I, des gènes DQβ, DRα, DRβ du CMH de classe II, et des gènes de la chaîne invariante. L'infection a cependant induit l'expression d'un transcript du gène DQα d'une taille inhabituelle de 1,5 kb, distinct du transcript de 1,3 kb induit par l'interféron γ. Bien que le rôle de ce transcript de 1,5 kb ne soit pas élucidé, cette observation suggère l'existence d'une stimulation de la synthèse d'ARNm à partir du gène DQα lors de l'infection par *Cowdria*, et le rôle possible des cellules endothéliales dans le processus de présentation d'antigènes aux lymphocytes $CD4^+$.

L'étude *in vitro* sur BBEC a été complétée par l'étude des mécanismes immunitaires développés par les ruminants lors des expérimentations de vaccination. L'évolution du phénotype des différentes sous-populations de cellules immuno-compétentes de chèvres et de bovins au cours du processus d'immunisation et d'épreuve virulente a été suivie par cytométrie en flux. Aucune variation significative des différentes populations cellulaires étudiées n'a été observée après les injections de primovaccination et de rappel. A l'inverse, après inoculation virulente, trois modifications importantes ont été observées dans l'ensemble des groupes vaccinés, aussi bien avec l'adjuvant de Freund qu'avec le Montanide ISA 50. Le nombre des lymphocytes T $CD4^+$, $CD8^+$ et $\gamma\delta$ a tendance à diminuer durant les 10 jours suivant l'injection virulente, ce qui conduit à l'apparition d'une lymphopénie. Pendant la même période, les monocytes augmentent fortement en nombre et prennent une morphologie de macrophages activés. La plus frappante des modifications est toutefois l'augmentation du nombre des lymphocytes $CD8^+$ qui atteint un maximum entre les jours 16 et 19 après inoculation virulente. A ce stade, ces cellules représentent plus de 50% des cellules mononuclées circulantes. Les analyses par double marquage ont montré que ces lymphocytes $CD8^+$ sont de type conventionnel $\alpha\beta$. L'augmentation considérable du nombre des lymphocytes $CD8^+$ suggère un rôle important de ces cellules dans la résistance à la cowdriose. Le pic de lymphocytes $CD8^+$ ne survient cependant que 2 semaines après l'infection virulente, alors que la plupart des animaux controles sont morts. D'autres mécanismes précédant la réponse $CD8^+$ doivent donc contribuer à la protection.

L'analyse de la réponse proliférative des cellules T avant l'épreuve virulente chez les bovins immunisés par des *Cowdria* tuées, a montré que tous les animaux répondent fortement *in vitro* à la stimulation par des lysats de *Cowdria*. La fraction de cellules qui prolifèrent est constituée de lymphocytes $CD4^+$ dont le degré d'activation est révélé par un haut niveau d'expression des antigènes de classe II du CMH et de récepteurs à l'IL-2. Des lignées de cellules T ont ainsi pu être générées *in vitro* par 3 stimulations hebdomadaires avec des *Cowdria* purifiées. Ces lignées se sont révélées sécréter des niveaux élevés d'interféron γ, et être constituées de 95 à 100% de lymphocytes $CD4^+$. L'utilisation de cellules présentatrices d'antigènes allogéniques et le blocage par des anticorps monoclonaux ont montré que cette prolifération était génétiquement et CMH classe II restreinte.

Il reste à démontrer que les lymphocytes $CD4^+$ sont activés *in vivo* après l'épreuve virulente qui a tendance à induire une lymphopénie, et que les $CD8^+$ ont une activité cytotoxique. Il faut noter également que l'ensemble des expérimentations ont été pratiquées avec des cellules mononuclées circulantes et que les résultats ne représentent peut-être pas fidèlement la réponse protectrice. Des études portant sur la lymphe drainant une lésion provoquée par des tiques infectées devraient permettre de compléter ces résultats.

Pour le développement de vaccins, l'appréciation de la diversité génétique et antigénique des microorganismes cibles est essentielle. Aucune méthode de typage fiable n'étant disponible pour *C. ruminantium*, nous avons développé une méthode RAPD (amplification de fragment d'ADN par réaction de polymérisation en chaîne à l'aide d'amorces aléatoires) qui nous a permis de montrer la grande diversité génétique de ce microorganisme. Sur 108 fragments d'ADN amplifiés par seize amorces différentes à partir du génome de six isolats (ou stocks) de *Cowdria*, 24% seulement se sont avérés communs entre les isolats. Le groupement des isolats en fonction de leur distance génétique a montré que la proximité génétique corrèle assez bien avec leur profil de protection immunitaire croisée. Celà demande cependant à être confirmé sur un plus grand nombre de stocks. L'hybridation de trois fragments d'ADN amplifiés par RAPD sur des blots d'ADN génomique de *Cowdria* a révélée un polymorphisme de type RFLP (polymorphisme de longueur des fragments de restriction) permettant comme le polymorphisme RAPD, de différencier les stocks entre eux.

Parallèlement aux études génétiques, une méthode de clonage de *Cowdria* basée sur des dilutions limites de cellules endothéliales infectées par une seule colonie a été développée. L'obtention de clones de ce micro-organisme était nécessaire pour les études d'immunologie, pour évaluer la diversité existant à l'intérieur d'un stock et entre les stocks de *Cowdria*, et pour étudier le phénomène d'atténuation de la virulence observé chez certains isolats.

SAMENVATTING

In het kader van dit promotieonderzoek zijn herkauwers gevaccineerd met verschillende antigeenpreparaten van *Cowdria ruminantium* en is vervolgens de opgewekte beschermende immuunrespons *in vitro* geanalyseerd. Daarnaast zijn methoden ontwikkeld om de genetische diversiteit van *C. ruminantium* te bepalen.

In twee achtereenvolgende proeven hebben we aangetoond dat twee injecties met geïnactiveerde elementaire deeltjes van *C. ruminantium* in Freunds-adjuvans geiten beschermden tegen een dosis *C. ruminantium* die lethaal is voor controledieren. Dit immunisatieprocédé bleek ook een doeltreffende bescherming te bieden voor runderen. Omdat het Freund-adjuvans in de praktijk niet gebruikt kan worden vanwege de nevenwerkingen, werd vervolgens een vergelijking gemaakt tussen de doeltreffendheid van twee vaccin- preparaten met een "water in olie" adjuvans bestaande uit elementaire deeltjes van *Cowdria* in fosfaatbuffer, met hetzij Freund-adjuvans hetzij Montanide ISA 50, een olie-adjuvans goedgekeurd voor het gebruik in dieren. Beide adjuvantia bleken even effectief te beschermen tegen een lethale dosis, drieëneenhalve maand na de vaccinatie. Eveneens werd de duur van de bescherming voor geiten bepaald door het vaccin op basis van Montanide ISA50. Drie van de 6 geiten of 5 van de 6 overleefden een infectie, repectievelijk 15 en 17 maanden na vaccinatie. Bij runderen werd het Freund-adjuvansvaccin getest. Alle 3 runderen werden beschermd, 10 maanden na vaccinatie. In totaal werd 64% van de gevaccineerde geiten (N = 31) en alle runderen (N = 5) beschermd, terwijl alle controledieren (30 geiten en 3 runderen) stierven.

Tijdens de dierproeven bleek dat het aantal virulente organismen invloed heeft op de incubatieperiode van de ziekte en op het overlevingspercentage van gevaccineerde dieren. Tijdens de vaccinatieproeven waren de incubatieperioden 6 $\pm$ 1,5 dag terwijl dit bij natuurlijke infectie gemiddeld zo'n 12 dagen is. Door de infectiedosis te verminderen tot 50 tot 10 TCLD50 (50% lethale dosis voor weefselcultuur) steeg de incubatieperiode tot 11 dagen. Het is dus nodig in de toekomst te komen tot een bepaling van de hoogte van deze dosis die een natuurlijke dosis zo dicht mogelijk benadert om de doeltreffendheid van vaccins te bepalen.

Vaccinatie met behulp van gedode bacteriën is een belangrijke verbetering ten opzichte van de methode van immunisatie door besmetting en antibiotica-

behandeling. De goede bescherming die werd verkregen en langdurige immuniteit bereikt door vaccinpreparaten met Montanide ISA50 betekent dat dit "eerste generatie vaccin" op grote schaal in het veld getest kan worden. Het onderzoek richt zich momenteel op het vinden van beschermende antigenen die verwerkt kunnen worden in een vaccin op recombinant-DNA basis.

In het tweede deel van het proefschrift worden studies beschreven over de pathologie en immuniteit van *Cowdria*-infecties, waarbij we ons speciaal gericht hebben op gevaccineerde herkauwers die vervolgens werden geïnfecteerd met levend virulent *Cowdria*.

Antilichamen van immune dieren zijn niet in staat endotheliale cellen in vitro te beschermen. Tesamen met de afwezigheid van bescherming voor dieren door de overdracht van hyperimmuun serum wijst dit op een overwegende rol van de cellulaire immuniteit.

Als eerste benadering werd een *in vitro* studie uitgevoerd op endotheliale cellen van bloedvaten in de hersenen van runderen (BBEC) die een doelwit voor *Cowdria* zijn. Deze studie heeft aangetoond dat de infectie van BBEC door *Cowdria* in synergie met een behandeling door het γ-interferon leidde tot de synthese van mRNA van IL-1, IL-6 en IL-8. Deze cytokines zouden verantwoordelijk kunnen zijn voor koorts en verhoogde doorlaatbaarheid van haarvaten waargenomen bij cowdriose. IL-8 zou betrokken kunnen zijn bij met mobiliseren van neutrofielen met mogelijk schadelijke gevolgen. Na besmetting met *Cowdria* trad geen verandering op in het transcriptieniveau van mRNA's voor MHC klasse I en van klasse II DQβ, DRα, DRβ. Wel leidde besmetting tot de expressie van een transcript van het gen DQα met een afwijkende lengte van 1.5 kb, dat duidelijk verschilde van het transcript van 1.3 kb geïnduceerd door het γ-interferon. Hoewel de rol van dit transcript van 1.5 kb niet is opgehelderd, suggereert het een stimulatie van de transcriptie van DQα mRNA tijdens besmetting met *Cowdria*, en de mogelijke rol van endotheliale cellen in het verloop van de presentatie van antigenen aan $CD4^+$ lymfocyten.

De *in vitro* studie op BBEC werd gecompleteerd met het bestuderen van verandering in de verschillende lymfocytensubpopulaties tijdens vaccinatie en challenge. Dit gebeurde door cytometrie. Er werden geen significante afwijkingen gevonden na vaccinatie en hervaccinatie. Maar na challenge traden drie belangrijke veranderingen op in alle gevaccineerde groepen. Het aantal T lymfocyten, zowel $CD4^+$ als $CD8^+$ en γδ, neemt af in de eerste 10 dagen, resulterend in een lymfopenie. In deze periode nam het aantal monocyten sterk

toe en kregen deze de morfologie van geactiveerde macrophagen. De meest opvallende verandering is zondermeer de toename van het aantal $CD8^+$ lymfocyten, van het $\alpha\beta$-type, die een maximum bereikt tussen dag 16 en 19 na challenge. In dit stadium maken ze meer dan 50% van de circulerende éénkernige cellen uit. De sterke toename van het aantal $CD8^+$ lymfocyten suggereert een belangrijke rol van deze cellen in de resistentie tegen cowdriose. De piek van $CD8^+$ lymfocyten trad echter pas 2 weken na infectie op, terwijl dan het merendeel van de controledieren al dood is. Andere beschermingsmechanismen voorafgaande aan de $CD8^+$-respons lijken dus bij te dragen aan de bescherming.

Ook T-helper lymfocyten blijken een rol te spelen in de immuunrespons op *Cowdria*-antigenen. Uit runderen gevaccineerd met geïnactiveerd *Cowdria* werden T-cellen geïsoleerd. Restimulatie *in vitro* met *Cowdria*-lysaten gaf een sterke immuunrespons. Deze was geheel het gevolg van een toename in het aantal $CD4^+$ lymfocyten. Deze cellen vertoonden een verhoging van het expressieniveau van klasse II MHC en de IL-2-receptor. T-cellijnen werden verkregen door *in vitro* drie maal te stimuleren met gezuiverd *Cowdria*-antigeen, met een week tussenpoos. Deze lijnen bestonden voor 95 tot 100% uit $CD4^+$ lymfocyten en bleken verhoogde niveaus γ-interferon af te scheiden. Geen proliferatie trad op met heteroloog antigeen en de proliferatie kon geblokkeerd worden door monoclonale antilichamen tegen MHC klasse II antigeen. Dit betekent dat deze proliferatie aan MHC klasse II restrictie onderhevig was.

Rest ons nog na te gaan of de $CD4^+$ lymfocyten ook door immunisatie worden geactiveerd na challenge met levend *Cowdria*, en of de $CD8^+$ T-cellen een cytotoxische werking hebben. Ook moet men zich realiseren dat alle experimenten zijn uitgevoerd met bloedcellen die anders zouden kunnen reageren dan bijv. cellen uit lymfeklieren in de omgeving van de tekenbeet.

Voor de ontwikkeling van vaccins is kennis nodig over de genetische en antigene diversiteit van de bestudeerde microorganismen. Aangezien er geen enkele betrouwbare typeringsmethode beschikbaar was voor *C. ruminantium* hebben we een RAPD-methode (amplificatie van DNA fragment door polymerase kettingreactie met behulp van willekeurig gekozen oligonucleotiden) ontwikkeld. Deze stelde ons in staat de genetische diversiteit van dit microorganisme te bepalen. Van de 108 geamplifieerde DNA fragmenten uit 6 *Cowdria* isolaten, waarbij gebruik werd gemaakt van 16 verschillende primers, bleek slechts 24% van de fragmenten gemeenschappelijk te zijn tussen de isolaten. De ordening van isolaten naar hun genetische verwantschap toonde aan dat deze redelijk goed

overeenkomt met hun kruisbeschermingsprofiel. Deze bevinding dient echter bevestigd te worden met een groter aantal isolaten. Hybridisatie van drie DNA fragmenten geamplificeerd door RAPD op Southern blots van genomisch DNA van *Cowdria* bracht een RFLP (lengte polymorfisme van restrictiefragmenten) aan het licht, dat het mogelijk maakte, net als bij het RAPD- polymorfisme, de isolaten onderling te onderscheiden.

Parallel aan de genetische studies hebben we een cloneringsmethode voor *Cowdria* ontwikkeld gebaseerd op de eindverdunning van endotheelcellen geïnfecteerd met één *Cowdria* colonie. Deze methode is belangrijk om de diversiteit tussen de isolaten en binnen eventuele mengisolaten van *Cowdria* verder te kunnen bestuderen.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Professor B.A.M. Van der Zeijst who accepted to be my promotor for this thesis. For his interest in *Cowdria* research, his advices and the time he spent in scientific and english corrections with frequent problems of communications between Guadeloupe and Utrecht, many thanks.

A special thanks to Dr F. Jongejan as a co-promotor of this thesis, but also and especially for our long and fruitful collaboration in cowdriosis research.

I am very greatful to all my colleagues from CIRAD-EMVT who collaborated in this program. Thanks also to my colleagues in Africa who make our research network on tick-borne-diseases of veterinary importance, so active.

Christian Sheikboudou and Isabelle Trap are thanked for their excellent technical assistance which was essential in all the experiments described in this work.

Finally, I would like to thank the European Union (Directorate General XII) for its financial support under TSD2, TSD3 and INCO-DC programs, and the Scientific Director of CIRAD-EMVT who supported the printing of the thesis.

Mes pensées vont à mes parents pour leur soutien de tous les jours, à mes frères et soeurs pour leur aide et leur complcité, à Chantal, Guillaume, Benjamin et Margaux dont la présence m'a permis de mener à bien ce travail.

CURRICULUM VITAE

Dominique Martinez werd geboren op 31 maart 1955 te Rabat, Marokko. In juni 1973 behaalde hij het Baccalauréat C in natuurkunde en wiskunde. Aansluitend begon hij met de studie diergeneeskunde aan de Ecole Nationale Vétérinaire de Maisons-Alfort. Deze studie sloot hij in 1979 af. Daarna deed hij een specialisatie in de tropische pathologie waarvoor hij in 1980 het certificaat ontving. In hetzelfde jaar behaalde hij zijn bevoegdheid tot veterinair inspecteur. Aan de Faculteit der Geneeskunde in Créteil werd in januari 1981 het veterinair doctoraal behaald. Vervolgens bekwaamde hij zich aan het Institut Pasteur in Parijs in de virologie en bacteriologie, welke specialisaties hij in respectievelijk december 1984 en april 1985 afsloot. In 1981 en 1982 was hij werkzaam als adviseur van de Directeur van Veterinaire Diensten in de Republiek Djibouti en had daarbij de leiding over een aantal diergezondheids-programma's. In juli 1982 kreeg hij een aanstelling aan het IEMVT in Bamako, Mali waar hij een epidemiologish onderzoek leidde naar runderpest. Door het IEMVT werd hij in 1983 aan het hoofd gesteld van een team dat, in samenwerking met het Institut Pasteur, in Mauretanië een epidemiologisch onderzoek uitvoerde bij kleine knaagdieren en kamelen. Van 1985 tot heden is hij werkzaam aan het CIRAD-EMVT in Guadeloupe als hoofd van het microbiologisch laboratorium, waar gewerkt wordt aan de ontwikkeling van vaccins voor cowdriose en dermatophilose. Het promotieonderzoek, beschreven in dit proefschrift, werd aan dit instituut uitgevoerd.

PUBLICATIONS

Publications in journals

1. Saluzzo, J. F., Digoutte, J. P., Chartier, C., Martinez, D., Bada, R. 1985. Focus of Rift Valley Fever Virus transmission in Mauritania. *Lancet* 1, 116.

2. Saluzzo, J. F., Camicas, J. L., Chartier, C., Martinez, D., Digoutte, J. P. 1986. Le virus de la fièvre hémorragique de Crimée-Congo (CCHF) en Mauritanie. *Cah. ORSTOM, Sér. Ent. méd. et Parasitol.* 24, 129-137.

3. Saluzzo, J. F., Chartier, C., Bada, R., Martinez, D., Digoutte, J. P. 1987. La fièvre de la Vallée du Rift en Afrique de l'Ouest. *Rev. Elev. Méd. Vét. Pays Trop.* 40, 215-223.

4. Barré, N., Matheron, G., Lefèvre, P. C., Le Goff, C., Rogez, B., Roger, F., Martinez, D., Sheikboudou, C. 1988. La dermatophilose des bovins à *Dermatophilus congolensis* dans les Antilles françaises. I. Caractéristiques des lésions et de la réponse sérologique. *Rev. Elev. Méd. Vét. Pays Trop.* 41, 129-138.

5. Barré, N., Matheron, G., Rogez, B., Roger, F., Martinez, D., Sheikboudou, C. 1988. La dermatophilose des bovins à *Dermatophilus congolensis* dans les Antilles françaises. II. Facteurs de réceptivité liés aux animaux. *Rev. Elev. Méd. Vét. Pays Trop.* 41, 339-345.

6. Martinez, D. 1989. Note sur la production de lait de dromadaire en secteur périurbain en Mauritanie. *Rev. Elev. Méd. Vét. Pays Trop.* 42, 115-116.

7. Martinez, D., Swinkels, J., Camus, E., Jongejan, F. 1990. Comparaison de trois antigènes pour le diagnostic de la cowdriose par immunofluorescence indirecte. *Rev. Elev. Méd. Vét. Pays Trop.* 43, 159-166.

8. Matheron, G., Barré, N., Roger, F., Rogez, B., Martinez, D., Sheikboudou, C. 1990. La dermatophilose des bovins à *Dermatophilus congolensis* dans les Antilles françaises. III. Comparaisons entre élevages infectés et indemnes. *Rev. Elev. Méd. Vét. Pays Trop.* 42, 331-347.

9. Martinez, D., Prior, P. 1991. Survival of *Dermatophilus congolensis* in tropical clay soils submitted to different water potentials. *Vet. Microbiol.* 29, 135-145.

10. Muller Kobold, A., Martinez, D., Camus, E., Jongejan, F. 1992. Distribution of heartwater in the Caribbean determined on the basis of detection of antibodies to the conserved 32-kilodalton protein of *Cowdria ruminantium*. *J. Clin. Microbiol.* 30, 1870-1873.

11. Camus, E., Martinez, D., Beauperthuy, L., Benderdouche, A., Coisne, S., Corbette, C., Denormandie, N., Garris, G., Harris, G., King, T., Lanoy, L., Louvet, L., Lunel, E., Montrose, M., Nisbett, B., Nyack, B., Robinson, J., Rouchosse, P., Swanston, G. B., Thiebot, B., Thye, G. D. 1993. Heartwater in Guadeloupe and in the Caribbean. *Rev. Elev. Méd. Vét. Pays Trop.* 46, 109-114.

12. Du Plessis, J. L., Bezuidenhout, J. D., Brett, M. S., Camus, E., Jongejan, F., Mahan, S., Martinez, D. 1993. The serodiagnosis of heartwater, a comparison of 5 tests. *Rev. Elev. Méd. Vét. Pays Trop.* 46, 123-129.

13. Gueye, A., Martinez, D., Mbengue, M., Dieye, T., Diouf, A. 1993. Epidémiologie de la cowdriose au Sénégal. II. Résultats de suivis séro-épidémiologiques. *Rev. Elev. Méd. Vét. Pays Trop.* 46, 449-454.

14. Martinez, D., Sheikboudou, C., Couraud, P. O., Bensaid, A. 1993. *In vitro* infection of bovine brain endothelial cells by *Cowdria ruminantium*. *Res. Vet. Sci.* 55, 258-260.

15. Martinez, D., Aumont, G., Moutoussamy, M., Gabriel, D., Tatareau, A. H., Barré, N., Vallée, F., Mari, B. 1993. Epidemiological studies on dermatophilosis in the Caribbean. *Rev. Elev. Méd. Vét. Pays Trop.* 46, 323-332.

16. Martinez, D., Coisne, S., Sheikboudou, C., Jongejan, F. 1993. Detection of antibodies to *Cowdria ruminantium* in the serum of domestic ruminants by indirect ELISA. *Rev. Elev. Méd. Vét. Pays Trop.* 46, 115-120.

17. Martinez, D., Mari, B., Aumont, G., Vidalenc, T. 1993. Development of a single dilution ELISA to detect antibody to *Dermatophilus congolensis* in goat and cattle sera. *Vet. Microbiol.* 34, 47-62.

18. Maillard, J. C., Martinez, D., Bensaid, A. 1994. Correlation between the resistance character to dermatophilosis in zebu Brahman cattle and certain BoLA class I and class II MHC haplotypes. *Animal Genetics*, 25 (supp.2), 18.

19. Martinez, D., Maillard, J. C., Coisne, S., Sheikboudou, C., Bensaid, A. 1994. Protection of goats against heartwater acquired by immunisation with inactivated elementary bodies of *Cowdria ruminantium*. *Vet. Immunol. Immunopathol.* 41, 153-163.

20. Bourdoulous, S., Bensaid, A., Martinez, D., Sheikboudou, C., Trap, I., Strosberg, A. D., Couraud, P. O. 1995. Infection of bovine brain microvessel endothelial cells with *Cowdria ruminantium* elicits interleukin-1beta, -6 and -8 mRNA production and expression of an unusual MHC class II DQalpha transcript. *J. Immunol.* 154, 4032-4038.

21. Van Vliet, A. H. M., Van der Zeijst, B. A. M., Camus, E., Mahan, S. M., Martinez, D., Jongejan, F. 1995. Use of a specific immunogenic region on the *Cowdria ruminantium* MAP1 protein in a serological assay. *J. Clin. Microbiol.* 33, 2405-2410.

22. Martinez, D., Perez, J.M., Sheikboudou, C., Debus, A., Bensaid, A. 1996. Comparative efficacy of Freund's and Montanide ISA 50 adjuvants for the immunisation of goats against heartwater with inactivated *Cowdria ruminantium*. *Vet. Parasitol.* 67, 175-184.

23. Totté, P., Mac Keever, D., Martinez, D., Bensaid, A. 1996. Analysis of T-cell responses in cattle immunized against heartwater by vaccination with killed elementary bodies of *Cowdria ruminantium*. Infect. Immun. 65, 236-241.

24. Totté, P., Vachiery, N., Martinez, D., Trap, I., Ballingdall, K. T., MacHugh, N. D., Bensaid, A., Werenne, J. 1996. Recombinant bovine interferon gamma inhibits the growth of *Cowdria ruminantium* but fails to induce MHC class II following infection of endothelial cells. *Vet. Immunol. Immunopathol.* 53, 61-71.

25. Katz J. B., DeWald, R., Dawson, J. E., Camus, E., Martinez, D., Mondry, R. 1997. Development and evaluation of a recombinant antigen, monoclonal antibody-based competitive ELISA for heartwater serodiagnosis. *J. Vet. Diagn. Invest.* (In press).

26. Mondry, R., Martinez, D., Camus, E., Liebisch, A., Van Vliet, A.H.M., Jongejan, F. 1997. Validation of the recombinant MAP1B Based indirect ELISA for the detection of antibodies to *Cowdria ruminantium* infection of livestock in the Caribbean. (Submitted)

27. Mondry, R., Martinez, D., Camus, E., Liebisch, A., Barbet, A., DeWald, R., Katz, J.B. 1997. Validation of the recombinant MAP1B Based competitive ELISA for the detection of antibodies to *Cowdria ruminantium* infection of livestock in the Lesser Antilles. (Submitted)

28. Perez, J.M., Martinez, D., Debus, A., Sheikboudou, C., Bensaid, A. 1997. Detection of genomic polymorphisms among isolates of the intracellular

bacterium *Cowdria ruminantium* by random amplified polymorphic DNA and Southern blotting. (submitted)

29. Perez, J.M., Martinez, D., Debus, A., Sheikboudou, C., Bensaid, A. 1997. Development of an *in vitro* cloning method for the rickettsia *Cowdria ruminantium* (submitted).

30. Vachiéry, N., Trap, I., Totté, P., Martinez, D., Bensaid, A. 1997. Inhibition of MHC class I and class II surface expression on bovine endothelial cells upon inecftion with *Cowdria ruminantium*. *Inf. Immun.* (submitted)

Book chapters

31. Martinez, D. 1990. Role of *Amblyomma variegatum* in the pathogenesis of severe clinical dermatophilosis. In C. Von Tscharner, & R. E. W Halliwell (Editors), (Veterinary Dermatology). (pp. 478). London: Baillière Tindall.

32. Martinez, D., Barré, N., Mari, B., & Vidalenc, T. 1992. Studies on the role of *Amblyomma variegatum* in the transmission of *Dermatophilus congolensis*. In B. Fivaz, T. Petney, & I. Horak (Editors), Tick vector biology. Medical and veterinary aspects. (pp. 87-99). Springer-Verlag.

33. Camus, E., Barré, N., Martinez, D., Uilenberg, G., 1996. Heartwater (cowdriosis), a review. Cirad-OIE, 177 p.

Congress

34. Saluzzo, J. F., Adam, F., Martinez, D., Camicas, J. L., Baudon, D., Chartier, C., Digoutte, J. P. 1985. Les fièvres hémorragiques d'origine virale, contribution à leur étude au Sénégal, en Mauritanie et au Burkina-Faso. In *25ème Conf. Techn. OCCGE*. Bobo-Dioulasso, Burkina-Faso, 15-19 avril 1985.

35. Martinez, D., Mari, B., Aumont, G., Vidalenc, T. 1990. Serodiagnosis of dermatophilosis by ELISA. Development of a one dilution test for epidemiological studies. In *IFS/FAO/IAEA workshop "Animal Disease Diagnostics in Latin America"*. Heredia, Costa-Rica, 22 october-2 november 1990.

36. Martinez, D., Mari, B., Aumont, G., Vidalenc, T. 1991. Detection of IgG antibodies to *Dermatophilus congolensis* in ruminant sera by ELISA. In Hunter

A.G. (Editor), *International Conference on the Application of Biotechnology to Livestock in Developing Countries*. Edimburgh, UK, 4-8 september 1989.

37. Martinez, D. 1991.Epidemiology of dermatophilosis in the Caribbean and prospect for control. In *2nd International Symposium on Dermatophilosis*. Vom, Nigeria, 4-8 november 1991.

38. Camus, E., Matheron, G., Martinez, D., Pépin, L., Bensaid, A. 1992. Methodological approaches to the study of resistance to cowdriosis and the possible role of cell-mediated immunity. in T. T. Dolan (Editor), *Recent development in the control of anaplasmosis, babesiosis and cowdriosis*. ILRAD, Nairobi (Kenya). pp. 93-98.

39. Bensaid, A., Camus, E., Depré, E., Maillard, J. C., Martinez, D., Matheron, G., Pépin, L., Ruff, G. 1993. Studies on the genetic resistance of native creole goats to cowdriosis and Brahman cattle to dermatophilosis. in G Uilenberg & R Hamers (Editors), *EEC Contractant Workshop on Resistance or Tolerance of Animals to Disease and Veterinary Epidemiology and Diagnostic Methods*. Rethymno, Crete (Greece). (pp. 30-34). CIRAD-EMVT. 2-6 november 1992.

40. Jongejan, F., Asselbergs, M., Camus, E., Kafuwa, P. T., Langa, A., Lawrence, J. A., Martinez, D., Muller Kobold, A. C., De Vries, N., Whiteland, A. P. 1993. Development of improved serodiagnosic methods for heartwater (*Cowdria ruminantium* infection) with emphasis on their aplication in endemic areas. in G Uilenberg & R Hamers (Editors), *EEC Contractant Workshop on Resistance or Tolerance of Animals to Disease and Veterinary Epidemiology and Diagnostic Methods*. Rethymno, Crete (Greece). (pp. 123-131). CIRAD-EMVT, 2-6 november 1992.

41. Martinez, D., Coisne, S., Sheikboudou, C., Moutoussamy, M., Mari, B., Vidalenc, T. 1993. The development of ELISA tests for the serodiagnosis of dermatophilosis and cowdriosis and their use in epidemiological studies. In G Uilenberg & Hamers R (Editors), *EEC Contractant Workshop on Resistance or Tolerance of Animals to Disease and Veterinary Epidemiology and Diagnostic Methods*. Rethymno, Crete (Greece). (pp. 123-131). CIRAD-EMVT, 2-6 november 1992.

42. Van Vliet, A. H. M., Mahan, S. M., Martinez, D., Camus, E., Van der Zeijst, B. A. M., Jongejan, F. 1994. Development of a *Cowdria ruminantium* specific ELISA based on recombinant antigens. In Paling R.W. & De Gooijer J.H.A. (Editors), *5th Symposium on Tropical Animal Health and Production, Application of biotechnology*. Utrecht, The Netherlands. (pp. 29-32), 30 september 1994.

43. Chesné, P., Cognié, Y., Martinez, D., Déprés, E., Aumont, G. 1995. Production of identical tropical goat twins for pathology research. 11th meeting of European Associations of Embryo Transfer (EAET), Hannover, 8-9 september 1995. p144.

44. Perez, J.M., Martinez, D., Debus, A., Sheikboudou, C., Bensaid, A. 1995. A method for cloning *Cowdria ruminantium* and analysis of its genetic diversity by RAPD. 2nd Tick Host Pathogen Interface (THPI) congress, Krüger Park, South Africa, 27-31 august 1995.

45. Maillard, J.C., Martinez, D., Bensaid, A. 1996. An amino acid sequence coded by the exon 2 of the BoLA DRB3 gene associated with a BoLA class I specificity constitutes a likely genetic marker of resistance to dermatophilosis in Brahman zebu cattle of Martinique (FWI). Ann. N. Y. Acad. Sci., 791, 185-197.

46. Van Vliet, A.H.M., Van der Zeijst, B.A.M., Camus, E., Mahan, S., Martinez, D., Jongejan, F. 1996. Recombinant expression and use in serology of a specific fragment from the *Cowdria ruminantium* MAP1 protein. Ann. N. Y. Acad. Sci., 791, 35-45.

Achevé d'imprimer
en France par

PARA GRAPHIC

31240 L'UNION (Toulouse)
Tél. 05 61.37.64.70
Dépôt légal : Mai 1997